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Synthesis and acetylcholinesterase inhibitory activity of polyhydroxylated sulfated steroids: Structure/activity studies

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ABSTRACT

Disulfated and trisulfated steroids have been synthesized from cholesterol and their acetylcholinesterase inhibitory activity has been evaluated. In our studies we have found that the activity was not only dependent on the location of the sulfate groups but on their configurations. 2β,3α,6α-trihydroxy-5α-cholestan-6-one trisulfate (**18**) was the most active steroid with an IC_{50} value of 15.48 μM comparable to that of 2β,3α-dihydroxy-5α-cholestan-6-one disulfate (**1**). Both compounds were found to be less active than the reference compound eserine. The butyrylcholinesterase activity of **1** and **18** was one magnitude lower than that against acetylcholinesterase revealing a selective inhibitor profile.

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1. Introduction

Alzheimer's disease (AD) is an age-related neurodegenerative process characterized by a progressive memory loss, decline in language skills and other cognitive impairments [1]. According to the cholinergic hypothesis, the selective and irreversible deficiency of cholinergic functions leads to memory impairment in AD [2]. Acetylcholine serves as a neurotransmitter in the central and peripheral nervous system. Acetylcholinesterase (AChE), located either in peripheral (muscles) and/or central nervous (cholinergic) system, stops the function of acetylcholine by its hydrolytic destruction in the cholinergic synapses [3]. Another cholinesterase, namely butyrylcholinesterase (BChE), is involved in the metabolic degradation of acetylcholine and differs from AChE in tissue distribution and sensitivity to substrates and inhibitors. The relative proportions of AChE to BChE depend on the brain regions and the stage of disease progression in AD patients [4]. BChE activity increases as AD progresses, which suggests that BChE may also play an important role in cholinergic dysfunction, particularly at the latter stages of AD. For this reason, the administration of inhibitors with different AChE/BChE selectivity should be very useful as AD progresses [5].

Enhancement of acetylcholine level in the brain is considered one of the most promising approaches for treating AD [6]. 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 and related conditions such as Lewy Body dementia, subcortical vascular dementia and Parkinson's disease [7,8].

The enhancement of the inhibitory activity against AChE by sulfation of calenduladiol, a pentacyclic triterpene isolated from *Chuquiraga erinacea*, (IC_{50} from 94 to 0.190 mM) [9] prompted us to test the acetylcholinesterase inhibitory activity of two sulfated compounds, disodium 2β,3α-dihydroxy-5α-cholestan-6-one disulfate (**1**) and disodium 2β,3α-dihydroxy-5α-cholestane disulfate (**2**), and compare it to their corresponding diols (**3** and **4**) (Fig. 1) [10,11]. The results were indicative of the role of the sulfate groups in the AChE inhibitory activity of **1** and **2** and showed that the presence of a carbonyl group at C-6 in **1** increases the activity [11]. In this report, we synthesized disulfated and trisulfated steroids with sulfate groups at C-2, C-3 and C-6 (α/β configurations) on ring A and B and determined their acetylcholinesterase inhibitory activity. Interestingly, we found that the activity of the sulfated steroids is not only dependent on the location of the sulfate groups but on their configurations. Our results provide new evidence for the relationship between chemical structure and acetylcholinesterase inhibitory activity.

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

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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 and on a Perkin Elmer Spectrum 400 with an ATR accessory. UV spectra were recorded on a JASCO V-630BIO 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 detected by spraying with sulfuric acid (10% H_2O). 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. Preparative HPLC was carried out on an SP liquid chromatograph equipped with a Spectra Series P100 solvent delivery system, a Rheodyne manual injector and a refractive index detector using a Phenomenex AQUA 5 μ C_{18} 125A column. All chemicals and solvents were analytical grade and solvents were purified by general methods before being used.

The commercially available trimethylamine-sulfur trioxide complex was purchased from Aldrich. 2β,3α-Dihydroxy-5α-cholestan6-one (**3**) was used as starting material for the synthesis of compounds **10**–**14** and 5α-cholest-2-en-6-one (**5**), to obtain the rest of the compounds (i.e. **6**–**9** and **15**–**18**). Compounds **3** and **5** were obtained following the procedure described in our previous work [11]. Acetylcholinesterase (electric eel), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), acetylthiocholine iodide (ATCI), butyrylthiocholine iodide (BTCI) and eserine were purchased from Sigma. Butyrylcholinesterase (horse serum) was purchased from MP Biomedicals.

2.2. 2β,3β-Dihydroxy-5α-cholestan-6-one (*6*)

Asymmetric dihydroxylation reactions were performed on 5α-cholest-2-en-6-one (**5**) using the experimental conditions reported by Sharpless [12]. A solution of **5** (50 mg, 0.13 mmol) in *tert*-butyl alcohol (2 ml) was added to a solution of AD-mix-α (181.7 mg) in water (2 ml). The mixture was stirred for few minutes at room temperature until two clear phases were observed. Methanesulphonamide (12.5 mg, 0.13 mmol) was added and the mixture was stirred at room temperature for two weeks. Then, a saturated sodium bisulphite solution (1.7 ml) was added to the cold reaction mixture and the suspension was stirred for 30 min at room temperature. After extraction with EtOAc (3 × 5 ml), the combined organic extracts were washed successively with 2 N KOH solution (3 × 5 ml) and water, dried over anhydrous $MgSO_4$ and evaporated to dryness. The reaction product was submitted to column chromatography on silica gel (0.063–0.200 mm), eluted with cyclohexane/EtOAc (60:40 v/v) and evaporated under reduced pressure to afford pure 2β,3β-dihydroxy-5α-cholestan-6-one (**6**)

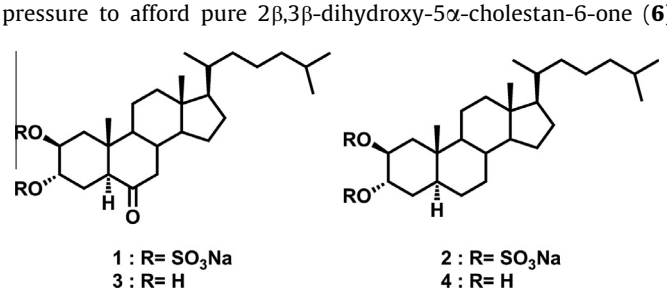


Fig. 1. Chemical structures of acetylcholinesterase inhibitors (**1**, **2**) and inactive diols (**3**, **4**).

(35.4 mg, 65%), m.p. 203–204 °C (acetone–H_2O) and 2α,3α-dihydroxy-5α-cholestan-6-one (**8**) (19 mg, 35%). ^{1}H NMR δ ($CDCl_3$): 0.66 (s, 3H, H-18), 0.86 (d, *J* = 6.6 Hz, 6H, H-26, H-27), 0.91 (d, *J* = 6.6 Hz, 3H, Me-21), 0.98 (s, 3H, H-19), 3.63 (dt, *J* = 11.7 Hz, 4.2 Hz, H-3α), 4.03 (m, 1H, H-2α). ^{13}C NMR δ ($CDCl_3$): 42.6 (C-1), 69.3 (C-2), 72.0 (C-3), 24.4 (C-4), 57.3 (C-5), 210.8 (C-6), 46.6 (C-7), 37.4 (C-8), 54.9 (C-9), 40.7 (C-10), 21.7 (C-11), 39.7 (C-12), 43.2 (C-13), 56.8 (C-14), 24.1 (C-15), 28.2 (C-16), 56.3 (C-17), 12.2 (C-18), 15.4 (C-19), 35.8 (C-20), 18.8 (C-21), 36.2 (C-22), 24.0 (C-23), 39.6 (C-24), 28.2 (C-25), 22.7 (C-26, C-27). Anal. calcd for $C_{27}H_{46}O_3.½H_2O$, C 75.82, H 11.08, O 13.10. Found, C 75.46, H 11.29. HREIMS (ESI+), calculated for $C_{27}H_{46}NaO_3$ [M + Na^+]: 441.3339, found *m/z* = 441.3322. FT-IR (NaBr, film, cm^{-1}) 3525, 3464 (ν O–H), 1696 (ν C=O), 1454 (δ_{as} CH_3), 1363 (δ_s CH_3).

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

Trimethylamine-sulfur trioxide complex (25.7 mg, 0.19 mmol) was added to a solution of 2β,3β-dihydroxy-5α-cholestan-6-one (**6**) (10 mg, 0.024 mmol) in DMF (1 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 (1 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 (85:15) afforded pure disodium 2β,3β-dihydroxy-5α-cholestan-6-one disulfate (**7**) (11.8 mg, 79.4%), m.p. 210–215 °C (decomp). ^{1}H NMR δ ($CDOD_3$): 0.71 (s, 3H, H-18), 0.88 (d, *J* = 6.6 Hz, 6H, H-26, H-27,), 0.95 (d, *J* = 6.4 Hz, 3H, Me-21), 1.00 (s, 3H, H-19), 4.31 (dt, *J* = 12.1 Hz, 3.7 Hz 1-H, H-3α), 4.84 (m, 1H, H-2α). ^{13}C NMR δ ($CDOD_3$): 41.7 (C-1), 76.5 (C-2), 78.3 (C-3), 23.6 (C-4), 58.3 (C-5), 213.2 (C-6), 47.1 (C-7), 38.8 (C-8), 55.5 (C-9), 42.0 (C-10), 25.0 (C-11), 40.9 (C-12), 44.2 (C-13), 57.7 (C-14), 24.9 (C-15), 29.2 (C-16), 57.5 (C-17), 12.4 (C-18), 15.6 (C-19), 37.1 (C-20), 19.1 (C-21), 37.3 (C-22), 22.7 (C-23), 40.7 (C-24), 29.2 (C-25), 22.9 (C-26), 23.2 (C-27). HREIMS (ESI−), calculated for $C_{27}H_{44}NaO_9S_2$ [M–Na]$^-$: 599.2330, found *m/z* = 599.2355. ATR FT-IR (ZnSe, cm^{-1}) 1699 (ν C=O), 1463 (δ_{as} CH_3), 1376 (δ_s CH_3), 1220 (δ_s S=O).

2.4. 2α,3α-Dihydroxy-5α-cholestan-6-one (*8*)

Asymmetric dihydroxylation reactions were performed on cholest-2-en-6-one (**5**) using the experimental conditions reported by Sharpless [12]. A solution of **5** (200 mg, 0.52 mmol) in *tert*-butyl alcohol (5.8 ml) was added to a solution of AD-mix-β (732 mg) in water (5.8 ml). The mixture was stirred for few minutes at room temperature until two clear phases were observed. Methanesulphonamide (49.3 mg, 0.51 mmol) was added and the mixture was stirred at room temperature for two weeks. Then, a saturated sodium bisulphite solution (6.6 ml) was added to the cold reaction mixture and the suspension was stirred for 30 min at room temperature. After extraction with EtOAc (3 × 15 ml), the combined organic extracts were washed successively with 2 N KOH solution (3 × 15 ml) and water, dried over anhydrous $MgSO_4$ and evaporated to dryness. The reaction product was submitted to column chromatography on silica gel (0.063–0.200 mm), eluted with cyclohexane/EtOAc (60:40 v/v) and evaporated under reduced pressure to afford pure 2α,3α-dihydroxy-5α-cholestan-6-one (**8**) (123.4 mg, 65%), m.p. 199–200 °C (acetone–H_2O) and 2β,3β-dihydroxy-5α-cholestan-6-one (**6**) (67.3 mg, 35%), together with 12.4 mg of **5**. ^{1}H NMR δ ($CDCl_3$): 0.66 (s, 3H, H-18), 0.74 (s, 3H, H-19), 0.85 (d, *J* = 6.7 Hz, 3H, H-26), 0.86 (d, *J* = 6.8 Hz, 3H, H-27), 0.91 (d, *J* = 6.5 Hz, 3H, Me-21), 3.75 (ddd, *J* = 11.8 Hz, 4.7 Hz, 3.2 Hz, H-2β), 4.03 (m, 1H, H-3β). ^{13}C NMR δ (CD_3OD): 40.3 (C-1), 68.4 (C-2), 68.5 (C-3), 26.4 (C-4), 50.9 (C-5), 212.5 (C-6), 46.9 (C-7), 37.8 (C-8), 53.9 (C-9), 42.7 (C-10), 21.3 (C-11), 39.5 (C-12), 43.1

(C-13), 56.8 (C-14), 24.1 (C-15), 28.2 (C-16), 56.2 (C-17), 12.2 (C-18), 13.7 (C-19), 35.8 (C-20), 18.8 (C-21), 36.2 (C-22), 24.0 (C-23), 39.6 (C-24), 28.1 (C-25), 22.7 (C-26), 23.0 (C-27). Anal. calcd for $C_{27}H_{46}O_3.½H_2O$, C 75.83, H 11.07, O 13.10. Found, C 75.86, H 11.14. HREIMS (ESI+), calculated for $C_{27}H_{46}NaO_3$ [M + Na^+]: 441.3339, found *m/z* = 441.3323. FT-IR (NaBr, film, cm^{-1}) 3348, 3523 (ν O–H), 1710 (ν C=O), 1463 (δ_{as} CH_3), 1380 (δ_s CH_3).

2.5. Disodium 2α,3α-dihydroxy-5α-cholestan-6-one disulfate (**9**)

Trimethylamine-sulfur trioxide complex (25.7 mg, 0.19 mmol) was added to a solution of 2α,3α-dihydroxy-5α-cholestan-6-one (**8**) (10 mg, 0.024 mm ol) in DMF (1 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 (1 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 (85:15) afforded pure disodium 2α,3α-dihydroxy-5α-cholestan-6-one disulfate (**9**) (11.6 mg, 77.6%), m.p. 195–208 °C (decomp). ^{1}H NMR δ ($CDOD_3$): 0.69 (s, 3H, H-18), 0.82 (s, 3H, H-19), 0.86 (d, *J* = 6.6 Hz, 3H, H-26), 0.87 (d, *J* = 6.6 Hz, 3H, H-27), 0.93 (d, *J* = 6.5 Hz, 3H, Me-21), 4.44 (ddd, *J* = 12.3, 4.7, 3 Hz, 1H, H-2β), 4.86 (m, 1-H, H-3β). ^{13}C NMR δ ($CDOD_3$): 39.6 (C-1), 75.4 (C-2), 76.4 (C-3), 25.9 (C-4), 52.3 (C-5), 214.3 (C-6), 47.4 (C-7), 39.2 (C-8), 55.0 (C-9), 43.7 (C-10), 24.9 (C-11), 40.8 (C-12), 44.2 (C-13), 57.8 (C-14), 25.0 (C-15), 29.2 (C-16), 57.4 (C-17), 12.4 (C-18), 14.0 (C-19), 37.0 (C-20), 19.1 (C-21), 37.3 (C-22), 22.4 (C-23), 40.7 (C-24), 29.2 (C-25), 22.9 (C-26), 23.2 (C-27). HREIMS (ESI−), calculated for $C_{27}H_{44}NaO_9S_2$ [M–Na]$^-$: 599.2330, found *m/z* = 599.2349. ATR FT-IR (ZnSe, cm^{-1}) 1702 (ν C=O), 1463(δ_{as} CH_3), 1379 (δ_s CH_3), 1218 (δ_s S=O).

2.6. 2β,3α,6β-Trihydroxy-5α-cholestane (**10**)

Three portions of 0.5 ml of a solution of 4.5 mg/ml of sodium borohydride in methanol were added to a solution of 19.5 mg (0.046 mmol) of 2β,3α-dihydroxy-5α-cholestan-6-one (**3**) in 1.25 ml of methanol and the mixture was stirred at room temperature overnight. After acidification with 0.5 M HCl solution, methanol was added and the mixture was evaporated to eliminate the $B(OMe)_3$. Then, water was added and the steroid was extracted with EtOAc (3 × 10 ml). The combined organic extracts were washed with water, dried over anhydrous $MgSO_4$, filtered and evaporated to dryness. The crude triol **10** was purified by solid phase extraction over silica gel (55 μm). Fractions eluted with cyclohexane/EtOAc (70:30) afforded pure 2β,3α,6β-trihydroxy-5α-cholestane (**10**) (16.8 mg, 87%), m.p. >250 °C (acetone–H_2O). ^{1}H NMR δ (CD_3OD): 0.72 (s, 3H, H-18), 0.88 (d, *J* = 6.6 Hz, 6H, H-26, H-27), 0.94 (d, *J* = 6.5 Hz, 3H, Me-21), 1.18 (s, 3H, H-19), 1.62 (m, 1H, H-5α), 3.79 (m, 1H, H-2α), 3.71 (m, H-6α), 3.86 (m, 1H, H-3β). ^{13}C NMR δ (CD_3OD): 42.6 (C-1), 72.2 (C-2), 71.4 (C-3), 30.1 (C-4), 43.1 (C-5), 72.9 (C-6), 40.7 (C-7), 31.3 (C-8), 57.7 (C-9), 37.1 (C-10), 24.9 (C-11), 40.4 (C-12), 43.9 (C-13), 56.7 (C-14)*, 25.3 (C-15), 29.3 (C-16), 57.7 (C-17)*, 12.6 (C-18), 18.3 (C-19), 37.0 (C-20), 19.2 (C-21), 37.4 (C-22), 21.8 (C-23), 40.7 (C-24), 29.1 (C-25), 22.9 (C-26), 23.2 (C-27). Anal. calcd for $C_{27}H_{48}O_3.3/4$ H_2O, C 74.69, H 11.49, O 13.82. Found, C 74.26, H 11.58. HREIMS (ESI+), calculated for $C_{27}H_{48}NaO_3$ [M + Na^+]: 443.3496, found *m/z* = 443.3479. ATR FT-IR (ZnSe, cm^{-1}) 3302 (ν O–H), 1463 (δ_{as} CH_3), 1379 (δ_s CH_3).

2.7. Disodium 2β,3α-dihydroxycholest-5-ene disulfate (**11**)

Trimethylamine-sulfur trioxide complex (52.6 mg, 0.38 mmol) was added to a solution of 2β,3α,6β-trihydroxy-5α-cholestane (**10**) (7.8 mg, 0.019 mmol) in DMF (0.4 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 (1 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 (85:15) afforded pure disodium 2β,3α-dihydroxycholest-5-ene disulfate (**11**) (4.4 mg, 38%), m.p. >250 °C (MeOH). ^{1}H NMR δ (DMSO-d_6): 0.63 (s, 3H, H-18), 0.83 (d, *J* = 6.6 Hz, 6H, H-26, H-27), 0.89 (d, *J* = 6.3 Hz, 3H, Me-21), 1.05 (s, 3H, H-19), 4.32 (m, 1H, H-2α), 4.40 (m, 1H, H-3β), 5.17 (m, 1H, H-6). ^{13}C NMR δ (DMSO-d_6): 32.4 (C-1), 72.7 (C-2), 72.2 (C-3), 37.1 (C-4), 139.4 (C-5), 120.6 (C-6), 31.2 (C-7), 30.7 (C-8), 49.8 (C-9), 36.2 (C-10), 20.2 (C-11), 39.2 (C-12), 42.3 (C-13), 56.1 (C-14), 23.7 (C-15), 27.6 (C-16), 55.3 (C-17), 11.15 (C-18), 18.3 (C-19), 35.0 (C-20), 18.5 (C-21), 35.5 (C-22), 23.0 (C-23), 38.7 (C-24), 27.2 (C-25), 22.1 (C-26), 22.6 (C-27). HREIMS (ESI−), calculated for $C_{27}H_{44}NaO_8S_2$ [M–Na]$^-$: 583.2381, found *m/z* = 599.2361.

2.8. Disodium 2β,3α,6β-trihydroxy-5α-cholestan-2β,3α-disulfate (**12**)

Two portions of 7 mg of sodium borohydride were added to a solution of 8.6 mg (0.014 mmol) of disodium 2β,3α-dihydroxy-5α-cholestan-6-one disulfate (**1**) in 0.40 ml of methanol at room temperature for 4 h. After acidification with 0.5 M HCl solution, methanol was added and the mixture was evaporated to eliminate the $B(OMe)_3$. The reaction product was purified by solid phase extraction over silica gel (55 μm). Fractions eluted with dichloromethane/methanol (85:15) afforded pure disodium 2β,3α,6β-trihydroxy-5α-cholestan-2β,3α-disulfate (**12**) (8.4 mg, 97%), m.p. >250 °C (decomp) (acetone–H_2O). ^{1}H NMR δ (DMSO-d_6): 0.64 (s, 3H, H-18), 0.83 (d, *J* = 6.6 Hz, 3H, H-26), 0.84 (d, *J* = 6.6 Hz, 3H, H-27), 0.89 (d, *J* = 6.6 Hz, 3H, H-21), 1.03 (s, 3H, H-19), 3.53 (m, 1H, H-6α), 4.30 (m, 1H, H-2α), 4.37 (m, 1-H, H-3β). ^{13}C NMR (DMSO-d_6): 39.0 (C-1), 73.2 (C-2), 73.4 (C-3), 27.0 (C-4), 41.5 (C-5), 69.7 (C-6), 39.9 (C-7), 29.7 (C-8), 54.8 (C-9), 35.1 (C-10), 20.3 (C-11), 39.6 (C-12), 42.2 (C-13), 55.9 (C-14), 23.9 (C-15), 27.8 (C-16), 55.7 (C-17), 11.9 (C-18), 17.2 (C-19), 35.2 (C-20), 18.6 (C-21), 35.7 (C-22), 23.2 (C-23), 38.8 (C-24), 27.4 (C-25), 22.7 (C-26), 22.4 (C-27). HREIMS (ESI−), calculated for $C_{27}H_{46}NaO_9S_2$ [M–Na]$^-$: 601.2486, found *m/z* = 601.2486. ATR FT-IR (ZnSe, cm^{-1}) 3317 (ν O–H), 1462 (δ_{as} CH_3), 1373 (δ_s CH_3), 1212 (δ_s S=O).

2.9. Trisodium 2β,3α,6β-trihydroxy-5α-cholestane trisulfate (**13**)

Trimethylamine-sulfur trioxide complex (13.5 mg, 0.097 mmol) was added to a solution of disodium 2β,3α,6β-trihydroxy-5α-cholestan-2β,3α-disulfate (**12**) (12.5 mg, 0.020 mmol) in DMF (0.25 ml). The reaction mixture was irradiated and stirred at 60 °C for an hour 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. Preparative HPLC (methanol/water (75:25 v/v)) of the crude product afforded pure trisodium 2β,3α,6β-trihydroxy-5α-cholestane trisulfate (**13**) (12.8 mg, 88%), m.p. >250 °C (decomp. at 85 °C). ^{1}H NMR δ (DMSO-d_6): 0.65 (s, 3H, H-18), 0.84 (d, *J* = 6.6 Hz, 6H, H-26, H-27), 0.89 (d, *J* = 6.4 Hz, 3H, Me-21), 0.95 (s, 3H, H-19), 4.06 (m, 1H, H-6α), 4.30 (m, 1H, H-2α), 4.37 (m, 1H, H-3β). ^{13}C NMR δ (DMSO-d_6): 36.6 (C-1), 73.0 (C-2), 73.2 (C-3), 26.7 (C-4), 41.2 (C-5), 75.6 (C-6), 38.9 (C-7), 30.0 (C-8), 54.5 (C-9), 35.0 (C-10), 20.3 (C-11), 39.8 (C-12), 42.2 (C-13), 55.9 (C-14), 23.9 (C-15), 27.8 (C-16), 55.7 (C-17), 12.0 (C-18), 16.9 (C-19), 35.2 (C-20), 18.6 (C-21), 35.7 (C-22), 23.1 (C-23), 38.8 (C-24), 27.4 (C-25), 22.4 (C-26), 22.7 (C-27). HREIMS (ESI-), calculated for $C_{27}H_{45}Na_2O_{12}S_3$

[M–Na]$^-$: 703.1874, found *m/z* = 703.1894. ATR FT-IR (ZnSe, cm^{-1}) 1463(δ_{as} CH_3), 1382 (δ_s CH_3), 1203 (δ_s S=O).

2.10. 2β,3α-Dihydroxy-5β-cholestan-6-one (**14**)

To a solution of 2β,3α-dihydroxy-5α-cholestan-6-one (**3**) (8.1 mg, 0.019 mmol) in 1.7 ml of ethanol under reflux, small pieces of Na were added until no 2β,3α-dihydroxy-5α-cholestan-6-one was detected by TLC. Then, 10 ml of 15% NaCl solution was added and the steroid was extracted with EtOAc (3 × 10 ml). The combined organic extracts were dried over anhydrous $MgSO_4$ and evaporated to dryness. The reaction mixture was purified by solid phase extraction over silica gel (55 μm) using cyclohexane/EtOAc (60:40) to give 6.6 mg (81.5%) of **14** as a white crystalline solid, m.p. 157–158 °C (acetone–H_2O). ^{1}H NMR δ ($CDCl_3$): 0.65 (s, 3H, H-18), 0.86 (d, *J* = 6.7 Hz, 6H, H-26, H-27), 0.89 (s, 3H, H-19), 0.91 (d, *J* = 6.5 Hz, 3H, Me-21), 2.19 (m, 1-H, H-5β), 3.43 (m, 1-H, H-3β), 3.65 (ddd,*J* = 12.5 Hz, 8.7 Hz, 4.0 Hz 1H, H-2α). ^{13}C NMR δ ($CDCl_3$): 32.7 (C-1), 70.5 (C-2), 75.3 (C-3), 41.9 (C-4), 59.0 (C-5), 213.2 (C-6), 42.7 (C-7), 36.9 (C-8), 41.1 (C-9), 42.0 (C-10), 20.8 (C-11), 39.4 (C-12), 42.9 (C-13), 56.6 (C-14), 23.7 (C-15), 27.9 (C-16), 55.9 (C-17), 11.7 (C-18), 23.0 (C-19), 35.5 (C-20), 18.4 (C-21), 35.8 (C-22), 23.6 (C-23), 39.3 (C-24), 27.8 (C-25), 23.5 (C-26), 22.3 (C-27). Anal. calcd for $C_{27}H_{46}O_3$. 1/4 H_2O, C 76.64, H 11.07, O 12.29. Found, C 76.38, H 10.58. HREIMS (ESI+), calculated for $C_{27}H_{46}NaO_3$ [M + Na]$^+$: 441.3339, found *m/z* = 441.3340. ATR FT-IR (ZnSe, cm^{-1}) 3454, 3376(ν O–H), 1690 (ν C=O), 1457 (δ_{as} CH_3), 1376 (δ_s CH_3).

2.11. 5α-Cholest-2-en-6α-ol (**15**)

To a solution of 5α-cholest-2-en-6-one (**5**) (79.9 mg, 0.21 mmol) in 16.5 ml of *n*-propanol under reflux, small pieces of Na were added until no cholest-2-en-6-one was detected by TLC. Then, 10 ml of 15% NaCl solution was added and the steroid was extracted with AcOEt (3 x 20 ml). The combined AcOEt extracts were dried over anhydrous $MgSO_4$ and evaporated to dryness. The reaction product was purified by dry column flash chromatography on silica gel using hexane/EtOAc (98:2) to give 72.3 mg (90%) of **15** as a white crystalline solid, m.p. 120–121 °C (acetone–H_2O). ^{1}H NMR δ ($CDCl_3$): 0.66 (s, 3H, H-18), 0.76 (s, 3H, H-19), 0.86 (d, *J* = 6.6 Hz, 6H, H-26, H-27), 0.91 (d, *J* = 6.5 Hz, 3H, H-21), 3.44 (dt, *J* = 10.9 Hz, 4.6 Hz, H-6β), 5.59 (m, 1H, H-2), 5.67 (m, 1H, H-3). ^{13}C NMR δ ($CDCl_3$): 40.1 (C-1), 125.7 (C-2), 125.3 (C-3), 25.9 (C-4), 48.8 (C-5), 71.8 (C-6), 41.5 (C-7), 34.4 (C-8), 53.7 (C-9), 35.6 (C-10), 21.0 (C-11), 40.0 (C-12), 42.7 (C-13), 53.4 (C-14), 24.3 (C-15), 28.3 (C-16), 56.3 (C-17), 12.1 (C-18), 13.2 (C-19), 35.9 (C-20), 18.8 (C-21), 36.3 (C-22), 24.0 (C-23), 39.7 (C-24), 28.2 (C-25). 22.7 (C-26), 23.0 (C-27). Anal. calcd for $C_{27}H_{46}O$, C 83.87, H 11.99, O 4.14. Found, C 83.50, H 12.27. HREIMS (ESI+), calculated for $C_{27}H_{46}NaO$ [M + Na]$^+$: 409.3441, found *m/z* = 409.3442. FT-IR (NaBr, film, cm^{-1}) 3270 (ν O–H), 3017 (ν C=C-H), 1468 (δ_{as} CH_3), 1382 (δ_s CH_3).

2.12. 2α,3α-Epoxy-5α-cholestan-6α-ol (**16**)

A 10% Na_2CO_3 solution (2.02 ml) was added to 5α-cholest-2-ene-6α-ol (**15**) (51.2 mg, 0.13 mmol) in CH_2Cl_2 (1.8 ml). The reaction mixture was stirred vigorously at 5 °C and *m*-chloroperbenzoic acid (74.3 mg, 0.43 mmol) in 0.8 ml of CH_2Cl_2 was added slowly. The mixture was stirred for 4 h at room temperature, and then the aqueous layer was extracted with CH_2Cl_2 (3 × 10 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 solid phase extraction over silica gel (55 μm). Fractions eluted with cyclohexane/EtOAc (90:10) afforded pure 2α,3α-epoxy-5α-cholestan-6α-ol (**16**) (37.2 mg, 71%), as a colorless syrup. ^{1}H NMR δ ($CDCl_3$): 0.64 (s, 3H, H-18), 0.77 (s, 3H, H-19), 0.86 (d, *J* = 6.6 Hz, 3H, H-26), 0.86 (d, *J* = 6.7 Hz, 3H, H-27), 0.90 (d, *J* = 6.6 Hz, 3H, Me-21), 3.12 (dd, *J* = 6.0 Hz, 4.0 Hz, 1H H-2β), 3.24 (m, 1H, H-3β), 3.38 (dt, *J* = 11.1 Hz, 4.7 Hz, H-6β). ^{13}C NMR δ ($CDCl_3$): 38.7 (C-1), 50.7 (C-2), 52.4 (C-3), 24.6 (C-4), 44.1 (C-5), 71.1 (C-6), 41.8 (C-7), 34.2 (C-8), 53.4 (C-9), 34.6 (C-10), 20.9 (C-11), 39.8 (C-12), 42.5 (C-13), 56.1 (C-14), 24.3 (C-15), 28.3 (C-16), 56.3 (C-17), 12.0 (C-18), 14.5 (C-19), 35.9 (C-20), 18.8 (C-21), 36.3 (C-22), 23.9 (C-23), 39.6 (C-24), 28.2 (C-25), 22.7 (C-26), 23.0 (C-27). HREIMS (ESI+), calculated for $C_{27}H_{46}NaO_2$ [M + Na]$^+$: 425.3390, found *m/z* = 425.3396. FT-IR (NaBr, film, cm^{-1}) 3406 (ν O–H), 1469 (δ_{as} CH_3), 1377 (δ_s CH_3), 1255 (ν_s C–O–C), 808 (ν_{as} C–O–C).

2.13. 2β,3α,6α-Trihydroxy-5α-cholestane (**17**)

A solution of epoxide **16** (34.8 mg, 0.086 mmol) in THF (1.77 ml) was treated with 1 N H_2SO_4 (0.05 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 (5 ml), and extracted with EtOAc (3 × 5 ml). The combined organic extracts were washed with water, dried over anhydrous $MgSO_4$, filtered and evaporated to dryness. The reaction product was purified by solid phase extraction over silica gel (55 μm). Fractions eluted with cyclohexane/EtOAc (40:60) afforded pure 2β,3α,6α-trihydroxy-5α-cholestane (**17**). (35.6 mg, 98%), m.p. 249–250 °C (acetone–H_2O). ^{1}H NMR δ ($CDCl_3$/CD_3OD; 4:1): 0.66 (s, 3H, H-18), 0.87 (d, *J* = 6.6 Hz, 6H, H-26, H-27), 0.98 (s, 3H, H-19), 0.91 (d, *J* = 6.5 Hz, 3H, Me-21), 3.81 (m, 1H, H-2α), 3.38 (dt, *J* = 11.0 Hz, 4.5 Hz, H-6β), 3.84 (m, 1H, H-3β). ^{13}C NMR δ ($CDCl_3$/CD_3OD; 4:1): 40.4 (C-1), 70.5 (C-2), 69.9 (C-3), 25.4 (C-4), 45.9 (C-5), 69.3 (C-6), 41.7 (C-7), 34.0 (C-8), 54.9 (C-9), 36.7 (C-10), 24.0 (C-11), 40.1 (C-12), 42.8 (C-13), 56.5 (C-14)*, 24.3 (C-15), 28.4 (C-16), 56.5 (C-17)*, 12.2 (C-18), 15.2 (C-19), 36.0 (C-20), 18.8 (C-21), 36.4 (C-22), 21.0 (C-23), 39.7 (C-24), 28.2 (C-25), 22.6 (C-26), 22.9 (C-27). (*) Signals might be interchangeable. Anal. calcd for $C_{27}H_{48}O_3$, C 77.09, H 11.50, O 11.41. Found, C 77.34, H 11.95. HREIMS (ESI+), calculated for $C_{27}H_{48}NaO_3$ [M + Na$^+$]: 443.3496, found *m/z* = 443.3494. FT-IR (NaBr, film, cm^{-1}) 3262 (ν O–H), 1460 (δ_{as} CH_3), 1377 (δ_s CH_3).

2.14. Trisodium 2β,3α,6α-trihydroxy-5α-cholestane trisulfate (**18**)

Trimethylamine-sulfur trioxide complex (23.8 mg, 0.17 mmol) was added to a solution of 2β,3α,6α-trihydroxy-5α-cholestane (**17**) (7.2 mg, 0.017 mmol) in DMF (0.61 ml). The reaction mixture was irradiated and stirred at 150 °C for 9 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 presure and purified by solid phase extraction over silica gel (55 μm). Fractions eluted with CH_2Cl_2/MeOH (85:15) afforded pure trisodium 2β,3α,6α-trihydroxy-5α-cholestane trisulfate (**18**) (9.4 mg, 76%), m.p. 189–192 °C (decomp.). ^{1}H NMR δ (DMSO-d_6): 0.62 (s, 3H, H-18), 0.84 (d, *J* = 6.6 Hz, 6H, H-26, H-27), 0.87 (s, 3H, H-19), 0.88 (d, *J* = 6.6 Hz, 3H, Me-21), 3.82 (dt, *J* = 10.9 Hz, 4.3 Hz, H-6β), 4.37 (m, 1H, H-3β), 4.45 (m, 1H, H-2α). ^{13}C NMR δ (DMSO-d_6): 37.5 (C-1), 72.4 (C-2), 72.3 (C-3), 23.7 (C-4), 43.8 (C-5), 74.0 (C-6), 38.9 (C-7), 33.4 (C-8), 54.1 (C-9), 35.9 (C-10), 20.4 (C-11), 39.5 (C-12), 42.2 (C-13), 55.9 (C-14), 23.8 (C-15), 27.8 (C-16), 55.6 (C-17), 11.9 (C-18), 14.7 (C-19), 35.2 (C-20), 18.5 (C-21), 35.6 (C-22), 23.1 (C-23), 38.9 (C-24), 27.4 (C-25), 22.4 (C-26), 22.7 (C-27). HREIMS (ESI−), calculated for $C_{27}H_{45}Na_2O_{12}S_3$

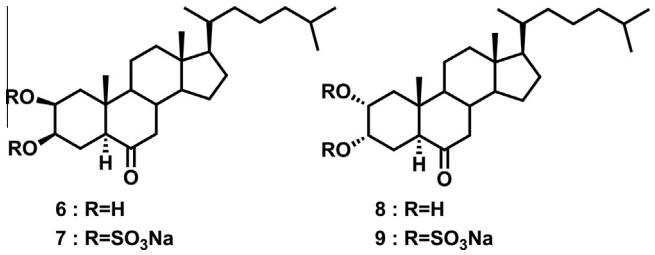


Fig. 2. Chemical structures of compounds **6** and **8** and their sulfated analogs (**7**, **9**).

[M–Na]−: 703.1874, found *m/z* = 703.1865. ATR FT-IR (cm^{-1}) 1485(δ_{as} CH_3), 1380 (δ_s CH_3), 1210 (δ_s S=O).

2.15. Cholinesterase inhibition assays

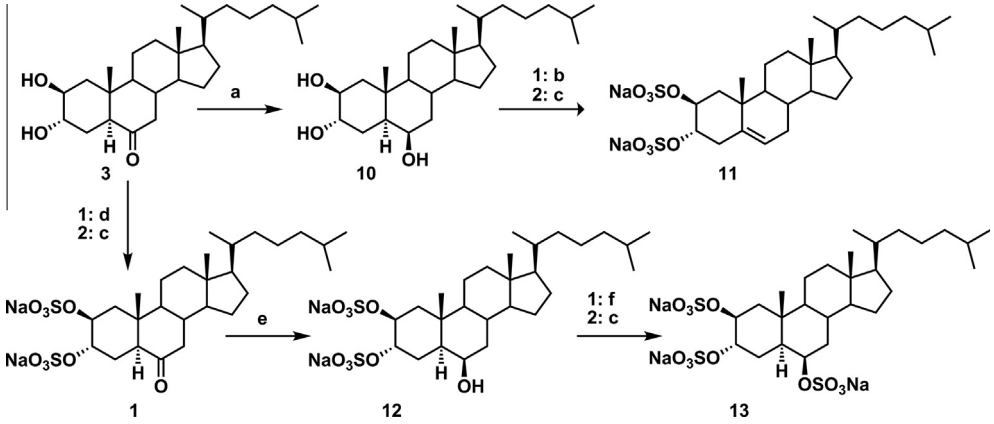
Electric eel (*Torpedo californica*) AChE and horse serum BChE were used as source of both cholinesterases. AChE and BChE inhibiting activities were measured in vitro by the spectrophotometric method developed by Ellman with slight modification [13]. The lyophilized enzyme, 500U AChE/300U BChE, was prepared in buffer phosphate 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 phosphateB (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. Compounds **6** and **8** required 2.5% of MeOH as cosolvent. Enzyme solution (300 μL) and 300 μL of sample solution 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%) was used as the reference AChE/BChE inhibitor.

3. Results and discussion

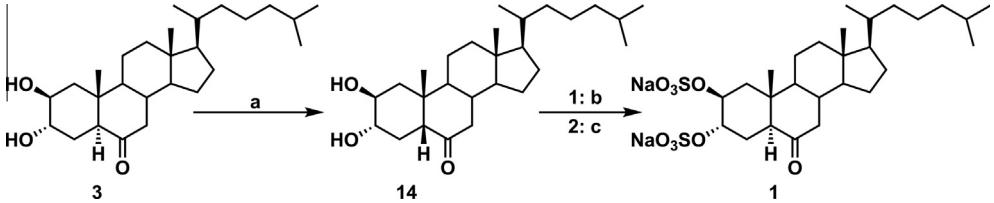
3.1. Chemistry

3.1.1. Synthesis of disodium (2α,3α)- and (2β,3β)-dihydroxy-5α-cholestan-6-one disulfate

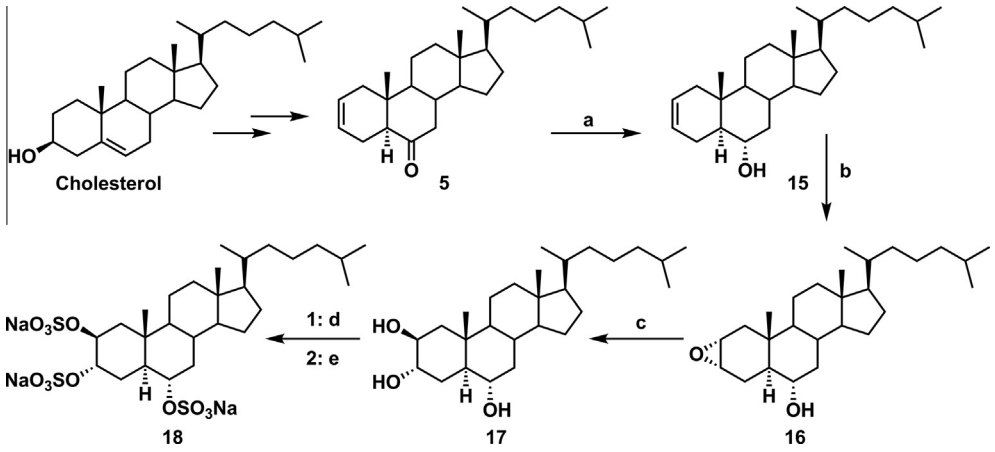
Isomeric disulfated steroids **7** and **9** (Fig. 2) were synthesized according to the following sequence. First, intermediate **5** was obtained from cholesterol in four steps as described previously [11]. Osmium-catalized asymmetric dihydroxylation [12] of double bond of **5** using $K_3Fe(CN)_6$ as cooxidant and hydroquinine-1,4-phthalazinediyl diether [$(DHQ)_2$-PHAL] as chiral ligand gave, after purification, 65% yield of the 2β,3β diol (**6**) and 35% of the 2α,3α diastereomer (**8**). When hydroquinidine-1,4-phthalazinediyl diether [$(DHQD)_2$-PHAL] was used as the chiral ligand, the 2α,3α diastereomer (**8**) was obtained in 51% yield together with **6** (28%) and starting material **5** (12%), which was recycled. These results are in accordance with reported synthesis of *cis*-diols using these hydroxylation reagents [14,15]. Subsequent treatment of diols **6** and **8** with 8 equiv. of trimethylamine-sulfur trioxide complex for 7 min at 150 °C at a microwave reactor [11] afforded the corresponding ammonium sulfates, which were transformed via ion exchange into the disodium salts **7** and **9**. The structures of **7** and **9** were confirmed by analysis of the proton and carbon NMR chemical shifts at C-2 and C-3. Resonances showing H-2α at 4.84 ppm (m) and H-3α at 4.31 ppm (dt, *J* = 12.1, 3.7 Hz) for compound **7** and H-2β at 4.44 ppm (ddd, *J* = 12.3, 4.7, 3 Hz) and H-3β at 4.86 ppm (m) for isomer **9** were characteristic of the presence of two sulfate groups at C-2 and C-3 in *cis* position. This was in accordance with the chemical shifts observed for C-2 (76.47 ppm (isomer **7**) and 75.44 ppm (isomer **9**)) and C-3 (78.30 ppm (isomer **7**) and 76.40 ppm (isomer **9**)) in the ^{13}C NMR spectra, as determined from the HSQC and HMBC spectra [16].



Scheme 1. Conditions: (a) $NaBH_4$, methanol, room temperature (b) 20 equiv. $Me_3N.SO_3$, DMF, 7 min, 150 °C, microwave (c) Amberlite CG-120 (MeOH) (d) 8 equiv. $Me_3N.SO_3$, DMF, 7 min, 150 °C, microwave (e) $NaBH_4$, methanol, room temperature (f) 5 equiv. $Me_3N.SO_3$, DMF, 1 h, 60 °C, microwave.



Scheme 2. Conditions: (a) Sodium, ethanol, reflux (b) 8 equiv. $Me_3N.SO_3$, DMF, 7 min, 150 °C, microwave (c) Amberlite CG-120 (MeOH).



Scheme 3. Conditions: (a) Sodium, *n*-propanol, reflux (b) *m*-ClPBA, Na_2CO_3, H_2O-Cl_2CH_2, room temperature, 4 h (c) H_2SO_4, THF room temperature, 24 h (d) 15 equiv. $Me_3N.SO_3$, DMF, 150 °C, microwave (e) Amberlite CG-120 (MeOH).

Table 1
Summary of the in vitro antiacetylcholinesterase activities.

Compound	IC_{50} (μM)
1	14.59 ± 0.88
3	59.65 ± 2.30
6	>200
7	40.94 ± 8.62
8	>200
9	30.66 ± 6.91
10	>200
11	>200
12	>200
13	>200
17	>200
18	15.48 ± 0.88
Eserine	0.0113 ± 0.0011

3.1.2. Synthesis of trisodium (2β,3α,6β)-trihydroxy-5α-cholestane trisulfate

The strategy followed to obtain compound **13** is shown in Scheme 1. The diol **3** was prepared from cholesterol in 6 steps as described previously [11]. Reduction of the carbonyl group with $NaBH_4$/MeOH from the α less hindered face of the steroid gave the 2β,3α,6β-triol (**10**) in 87% yield. The β configuration of the hydroxyl group at C-6 was inferred from NOE interactions between signals at 1.62 ppm (H-5α) and 3.71 (H-6α) ppm. Further sulfation of **10** with 20 equiv. of trimethylamine-sulfur trioxide complex for 7 min at 150 °C at a microwave reactor and elution through Amberlite CG-120 (sodium form) afforded a mixture of products, as observed by TLC. Purification of the mixture gave disulfated steroid **11** in 34% yield. The ^{1}H NMR spectrum of **11** showed a multiplet at 5.17 ppm (H-5) and two olefinic carbons at 139.4 (C-5) and 120.6 ppm (C-6), characteristic of a Δ^5 steroid [17]. This was confirmed by correlation of the C-19 methyl signal at 18.40 ppm with the multiplet at 5.17 ppm in the HMBC spectrum. In order to avoid the elimination of the axial hydroxyl group at C-6, we developed a new strategy based on sulfation of the diol **3** [11], reduction of the carbonyl group, and further sulfation of the hydroxyl at C-6 in milder conditions (Scheme 1). In this way, reduction of the disulfated ketone **1** with $NaBH_4$/MeOH, sulfation of the C-6 (β) hydroxyl of **12** with 5 equiv. of trimethylamine-sulfur trioxide complex for 1 h at 60 °C at a microwave reactor and transformation via ion exchange into the sodium salts afforded trisulfated triol **13** in 89% yield from **1**. The ^{1}H NMR spectrum of **13** showed a broad singlet at 4.06 ppm for H-6β, as confirmed by NOESY correlations.

3.1.3. Synthesis of trisodium (2β,3α,6α)-trihydroxy-5α-cholestane trisulfate

Reduction of **3** with sodium in ethanol under reflux gave the epimer at C-5 (**14**) instead of the expected 2β,3α,6α-triol (Scheme 2). The ^{13}C NMR spectrum of **14** showed a C-19 methyl signal at 23.0 ppm indicative of a *cis* conformation for rings A and B [17]. This was confirmed by NOESY correlations of the signal of H-5β at 2.19 ppm with CH_3-19 and H-3β. Evidently, epimerization at C-5 *via* enolate formation was favored over reduction of the carbonyl group due to the pseudo-equatorial position of both hydroxyl groups in **14**. On the other hand, sulfation of **14** in the same conditions as for **3** gave the disulfated steroid **1** in 84.1% yield.

Looking forward to prepare the 2β,3α,6α-triol **17** we followed the strategy of Murphy et al. [18] for the synthesis of trisulfated analog **18** of sokotrasterol sulfate, a natural product isolated from the sponge *Topsentia ophirhaphidites* (Scheme 3). Reduction of the ketone **5** with sodium in *n*-propanol (reflux) gave 5α-cholest-2-en-6α-ol (**15**). Further epoxidation and acid-catalyzed epoxide ring opening of **16** gave triol **17**. The ^{1}H NMR spectrum of **17** showed two multiplets at δ 3.81 (H-2α) and 3.84 (H-3β) ppm, geminal to hydroxyl groups at C-2 (70.47 ppm) and C-3 (69.93 ppm) and a double triplet at 3.38 ppm (H-6β) (J = 11.0 Hz, 4.5 Hz), as determined by analysis of ^{13}C NMR, HSQC, HMBC and NOESY spectra. Sulfation of **17** with 15 equiv. of trimethylamine-sulfur trioxide complex for 9 min at 150° in microwave reactor and transformation via ion exchange afforded trisodium 2β,3α,6α-trihydroxy-5α-cholestane trisulfate (**18**) in 76% yield (Scheme 3).

3.1.4. Evaluation of the acetylcholinesterase inhibitory activity

The acetylcholinesterase inhibitory activity of compounds **6–13**, **17** and **18** was evaluated and compared to that of analogs **1** and **3** (Table 1) previously synthesized by our group [10–11]. The compounds were found to be less active than the reference compound eserine. From the data shown in Table 1, *cis* disulfated compounds **7** and **9** were less active than isomer **1**, suggesting the importance of the sulfate configurations at C-2 and C-3 on the inhibitory activity. As reported previously [11], steroid **1** binds reversibly to the enzyme-substrate complex, yielding an inactive complex. This affinity is favored by hydrogen bonding interactions, which involve both sulfates at ring A. Presumably, a change in sulfate configurations at C-2 and C-3, as in isomers **7** and **9**, decreases their affinity to the enzyme-substrate complex. Interestingly, 2β,3α, 6α-trisulfated steroid **18** was the most active compound with an IC_{50} value of 15.48 μM comparable to that of compound **1** (14.59 μM). On the other hand, compound **13** with a sulfate group at C-6 (β) showed no inhibitory activity (IC_{50} > 200 μM) as well as

Table 2
Summary of the in vitro antibutyrylcholinesterase activities.

Compound	IC_{50} (μM)	Ratio IC_{50} BChE/AChE
1	>200	>13.7
18	176.10 ± 1.29	11.4
Eserine	0.0143 ± 0.0013	1.27

disulfated analogs **11** and **12** and alcohols **6**, **8**, **10** and **17**. These results indicate that the presence of *trans* diaxial sulfate groups at C-2 and C-3 together with a carbonyl or sulphate group at C-6 (α) increase the inhibitory activity.

In order to determine the AChE/BChE selectivity of the most active compounds **1** and **18**, their butyrylcholinesterase activity was evaluated. As shown in Table 2, the butyrylcholinesterase activity of **1** and **18** was one magnitude lower than that against AChE, revealing a selective inhibitor profile. 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 [19,20].

Acknowledgments

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