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Synthesis and antifungal activity of oxygenated cholesterol derivatives

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Abstract

A series of oxygenated cholesterol derivatives were prepared from new synthetic methods and evaluated for their in vitro antimicrobial properties against human pathogens. The activity was highly dependent on the structure of the different compounds involved. The best results were obtained with hydroxy ketones **2**, **4** and **5** and diketone **7** exhibiting activities against *S. cerevisiae* (ATCC 28383) and *Candida albicans* (CIP 1663-86). For example, compound **2** exhibited high activities against *C. albicans* (CIP 1663-86) and Amphotericine B and miconazole resistant strain *C. albicans* (CIP 1180-79) at a concentration of 1.5 μg/mL.

Keywords: Oxygenated sterols; Antifungal activity; Antimicrobial activity

1. Introduction

Oxygenated sterols, including both autoxidation products and sterol metabolites, have many important biological activities mostly related to the physiological control of cholesterol biosynthesis [1,2]. Several investigators have demonstrated that oxygenated cholesterol such as 7-ketocholesterol and 25-hydroxycholesterol inhibit the activity of β-hydroxy-β-methylglutaryl CoA (HMG CoA) reductase, the rate-limiting enzyme in the biosynthesis of cholesterol, in various in vitro test system [1,3–6]. In 1995, two 4,4-dimethyl-$\Delta^{8,24}$-sterols called FF-MAS (Follicular Fluid-Meiosis Activating Sterol) were found to have a regulatory function in meiosis [7] and since then numerous studies dealing with the design, synthesis and derivation of structure-activity relation ships of FF-MAS related sterol compounds have been reported [8–10]. Nevertheless, few studies have been devoted to the possible antifungal and antimicrobial activities against Gram-positive, Gram-negative bacteria and yeast of such oxygenated sterols. Ourisson et al. were the first to report preliminar results on the cytotoxity of such compounds towards tumor cells [11–13]. In this area, recent results have been reported on the synthesis and antiproliferative, anti-HIV and anti-Asthma properties of various oxygenated sterol derivatives [14–16]. In continuation of our work on biologically active sterol derivatives [17–22], we report herein the synthesis of various new oxygenated cholesterol derivatives and their promising antifungal properties since no biological activities of such derivatives were described to date, even if numerous similar natural compounds possessing interesting biological activities have been isolated.

2. Experimental section

All solvents were purified according to reported procedures, and reagents were used as commercially available. Tetrahydrofuran (THF) was distilled from sodium-benzophenone ketyl immediately prior to use. Ethylacetate and petroleum ether (35–60 °C) were purchased from SDS and used without any further purification. Column

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chromatography was performed on SDS silica gel (70–230 mesh). ^{1}H NMR and ^{13}C NMR spectra were recorded in $CDCl_3$ on a Bruker AC 300 spectrometer working at 300.00 and 75 MHz, respectively (the usual abbreviations are used: s: singulet, d: doublet, t: triplet, q: quadruplet, m: multiplet). Tetramethylsilane was used as internal standard. All chemical shifts are given in ppm.

2.1. Synthesis of 7-keto cholesterol 2

Compound **2** was prepared using a modified procedure previously described by Kinney et al. [23,24]. Cholesterol (50 g, 0.129 mol) and *N*-hydroxyphthalamide (22 g, 0.135 mol) were dissolved in EtOAc-acetone (1.5 L, 1:1 v/v) in a 2 L glass reactor equipped with a condenser and a mechanical stirrer. Benzoyl peroxide (3 g) was added at 50–60 °C. Air was bubbled into the reaction solution and stirring was maintained for 72 h at 50–60 °C. Additional 50/50 EtOAc-acetone was added to the reaction as needed to replenish what was lost due to air flow through the system. The reaction was followed by TLC on silicagel (50% EtOAc in petroleum ether) and judged completed after 72 h. After evaporation of all the solvents in vacuo, petroleum ether was added and the organic phase was washed with sodium carbonate solution until no orange coloration was observed. The organic layers were washed with brine and dried over $MgSO_4$. The solvent was removed and the sterol dissolved in pyridine (200 mL). The pyridine solution was cooled to 0 °C and $CuCl_2$ (1 g) was added. The solution was stirred overnight allowing the solution to warm to room temperature. After addition of water (200 mL), the solution was extracted with EtOAc (3×150 mL). The organic layer was washed with saturated $CuSO_4$ solution until no trace of pyridine was observed. The organic layer was washed with a 0.1 M HCl solution, dried over $MgSO_4$ and concentrated in vacuo. The oily residue was purified by chromatography on a silicagel column using EtOAc/petroleum ether as eluent (50/50) affording the expected hydroxy ketone **2** in 62% yield.

White solid; mp: 116 °C; ^{1}H NMR: $\delta = 5.45$–5.75 (m, 1H), 4.34 (s, 1H), 3.47–3.75 (m, 1H), 0.45–2.12 (m, 41H); ^{13}C: $\delta = 202.81, 165.59, 126.49, 70.89, 55.17, 50.34, 45.80, 43.49, 39.87, 38.67, 36.57, 36.11, 28.40, 26.72, 24.22, 23.22, 22.96, 21.61, 19.26, 17.71, 12.37$. $C_{27}H_{44}O_2$ calcd C 80.9, H 11.1; found C 81.0, H 10.8.

2.2. Synthesis of 7β-hydroxy cholestanol 3

In a 250 mL two necked round flask were placed under argon anhydrous THF (40 mL) at −78 °C and 40 mL of ammonia. Lithium wire (0.7 g, 0.14 mol) was added to the solution with vigorous stirring. Once the lithium was completely dissolved, 7-ketocholesterol **2** (3.5 g, 8.75×10^{-3} mol) was dissolved in 50 mL of anhydrous THF and added to the flask in a steady stream from a 100 mL addition funnel. The reaction was stirred for 3 h at −78 °C before being quenched by the addition of MeOH until no blue coloration was observed. The ammonia was allowed to evaporate overnight at room temperature. The residue was dissolved in 100 mL of a toluene/EtOAc solution. The organic layer was successively washed with a 0.1 N HCl solution, distilled water and brine. The organic layer was dried over Na_2SO_4 and concentrated in vacuo. The oily residue was purified by chromatography on a silicagel column using EtOAc/petroleum ether as eluent (1/4–1/1) affording the expected diol 3 in 74% yield.

White solid; mp: 175 °C; ^{1}H NMR: $\delta = 3.95$–4.30 (m, 1H), 0.40–3.45 (m, 47H); ^{13}C: $\delta = 75.21, 71.13, 55.80, 55.31, 52.55, 43.66, 43.48, 42.11, 40.05, 39.56, 38.13, 36.97, 36.26, 35.74, 35.00, 31.65, 31.50, 28.78, 28.07, 26.97, 23.91, 22.88, 22.62, 21.51, 18.86, 14.19, 12.51, 12.23$. $C_{27}H_{48}O_2$ calcd C 80.2, H 11.9; found C 80.4, H 10.9.

2.3. Synthesis of 7β-hydroxy cholestanone 4

A suspension of 7β-hydroxy cholestanol **3** (1 g, 1.9×10^{-3} mol) and silver carbonate on Celite (1 g) in toluene (70 mL) was stirred under argon at reflux overnight. The reaction mixture was filtered through a column of Florisil and the filtrate was concentrated in vacuo. The residue was purified by chromatography on a silicagel column using petroleum ether/ethylacetate (100/0 (100 mL), 50/50 (100 mL), 0/100 (300 mL)) as eluent affording the expected product **4** in 82% yield.

White solid; mp: 180 °C; ^{1}H NMR: $\delta = 3.05$–3.55 (m, 1H), 2.2–2.6 (m, 3H), 0.4–1.92 (m, 42H); ^{13}C: $\delta = 211.55, 74.66, 55.61, 55.24, 51.83, 44.18, 43.94, 43.66, 39.90, 39.54, 38.12, 35.72, 35.16, 28.75, 28.06, 23.89, 22.87, 22.62, 21.80, 18.84, 12.22, 11.63$. $C_{27}H_{46}O_2$ calcd C 80.5, H 11.5; found C 81.6, H 11.3.

2.4. Synthesis of 7α-hydroxycholestanone 5 and 3,7-cholestanedione 6

In a 250 mL two necked round flask were placed under argon at −78 °C 7-ketocholesterol **2** (300 mg, 7.46×10^{-4} mol) dissolved in anhydrous THF (10 mL). L-Selectride (2 equivalents) were slowly added at −78 °C and stirred for 5 h before being quenched by the addition of H_2O_2 and a solution of $NaHCO_3$ (10 mL). The residue was dissolved in 100 mL of ethylacetate, washed with brine and dried over $MgSO_4$. After filtration and evaporation of the solvents, the crude residue of 7α-hydroxy cholestanol (196 mg, 4.85×10^{-4} mol) and silver carbonate on Celite (631 mg) in toluene (70 mL) was stirred under argon at reflux overnight. The reaction mixture was filtered through a column of Florisil and the filtrate was concentrated in vacuo. The residue was purified by chromatography on a silicagel column using petroleum ether/ethylacetate (70/30 → (80/20)) as eluent affording the expected products **5** and **6** in, respectively, 13 and 17% yield.

Compound **5**: white solid; mp: 115 °C; ^{1}H NMR: $\delta = 3.3$–3.85 (m, 1H), 0.60–2.65 (m, 45H); ^{13}C: $\delta = 211.36$,

71.08, 57.18, 56.53, 54.33, 47.13, 39.86, 38.32, 37.07, 36.49, 36.08, 31.10, 30.44, 28.43, 28.39, 24.38, 24.20, 23.20, 22.93, 21.92, 19.03, 13.53, 12.40. $C_{27}H_{46}O_2$ calcd C 80.5, H 11.5; found C 80.7, H 10.8.

Compound 6: white solid; mp: 181 °C; ^{1}H NMR: δ = 0.8–2.8 (m, 41H), 0.6–0.775 (m, 3H); ^{13}C: δ = 211.66, 209.52, 57.91, 57.01, 56.52, 53.88, 47.02, 39.85, 39.79, 38.51, 38.43, 37.78, 37.39, 36.46, 36.08, 28.42, 28.39, 24.40, 24.19, 23.19, 22.93, 22.07, 19.02, 12.95, 12.41. $C_{27}H_{44}O_2$ calcd C 80.9, H 11.1; found C 81.0, H 10.8.

2.5. Synthesis of cholest-4-ene-3,6-dione 7

In a 250 mL two necked round flask cholesterol (5 g, 1.29×10^{-2} mol) was dissolved in anhydrous dichloromethane (50 mL) and pyridinium chlorochromate (8.34 g, 3.87×10^{-2} mol) was added. The mixture was stirred at room temperature for 3 days. Then an additional portion of pyridinium chlorochromate (4.2 g, 1.93×10^{-2} mol) was added. After further stirring at room temperature for 1 day, dry diethylether (150 mL) was added and the liquid was decanted from a brown gum. The insoluble residue was washed three times with dry diethylether (3×50 mL). The combined organic layers were washed with water, dried over $MgSO_4$, passed through a pad of Florisil and concentrated in vacuo. The residue was purified by chromatography on a silicagel column using petroleum ether/ethylacetate (5/2) as eluent affording the expected cholest-4-ene-3,6-dione **7** in 72% yield.

White solid; mp: 119 °C; ^{1}H NMR: δ = 7.15 (s, 1H), 0.55–2.72 (m, 41H); ^{13}C: δ = 202.38, 199.53, 161.13, 125.48, 56.59, 56.00, 51.01, 46.85, 42.58, 39.85, 39.50, 36.11, 35.72, 34.25, 34.02, 28.05, 24.01, 23.84, 22.86, 22.60, 20.92, 18.69, 17.55, 11.93. $C_{27}H_{42}O_2$ calcd C 81.4, H 10.6; found C 81.2, H 10.8.

2.6. Synthesis of cholest-5-ene-3,7-dione 8

Compound 8 was prepared using a modified procedure previously described by Corey et al. for the catalytic oxidation of secondary alcohols to ketones [25].

Into a 25 mL round-bottom flask, which had been flame dried and filled with argon, was placed CH_2Cl_2 (2 mL) and a dark orange CCl_4 solution of a cyclic chromate ester (3.0×10^{-3} mol) prepared from 2,4-dimethylpentane-2,4-diol and chromium trioxide. After cooling the mixture to 0 °C in an ice bath a solution of peroxyacetic acid (3 mL) and cholesterol (235 mg, 0.61 mmol) dissolved in 2 mL of dry CH_2Cl_2 were slowly added. The mixture was stirred at 0 °C for 48 h. Removal of the solvents in vacuo afforded a oily brown residue which was purified by chromatography on a silicagel column using petroleum ether/ethylacetate (75/25) as eluent affording the expected cholest-5-ene-3,7-dione **8** in 45% yield.

Red solid; mp: 91 °C; ^{1}H NMR: δ = 5.90 (s, 1H), 3.10 (s, 2H), 2.27–2.77 (m, 2H), 1.75–2.20 (m, 5H), 0.50–1.71 (m, 32H); ^{13}C: δ = 202.76, 199.94, 161.50, 125.83, 56.94, 56.35, 51.37, 47.20, 42.92, 40.20, 39.85, 36.45, 36.06, 34.59, 28.39, 24.18, 23.20, 22.94, 21.26, 19.03, 17.98, 12.27. $C_{27}H_{44}O_2$ calcd C 80.9, H 11.1; found C 81.0, H 10.8. $C_{27}H_{42}O_2$ calcd C 81.4, H 10.6; found C 81.2, H 10.7.

2.7. Synthesis of 7α-hydroxycholesterol 9

To a stirred and cooled (−78 °C) solution of 7-keto cholesterol 2 (300 mg, 6.78×10^{-4} mol) in dry THF (10 mL) was added a 1 M solution of K-Selectride in THF (2 mL, 2×10^{-3} mol). The mixture was stirred overnight allowing the solution to warm to room temperature. The organoborane was oxidized by addition of H_2O_2 until the color of the solution has disappeared and treated by a 1 N sodium hydroxide solution (2 mL). Toluene (40 mL) was added and the organic layer was successively washed with a solution of 1 N HCl, Na_2CO_3 then dried over $MgSO_4$. After filtration and removal of the solvents, the residue was purified by chromatography on a silicagel column using petroleum ether/ethylacetate (75/25) as eluent affording the expected 7α-hydroxycholesterol **9** in 62% yield.

White solid; mp: 185 °C; ^{1}H NMR: δ = 5.60–5.71 (m, 1H), 3.80–3.95 (m, 1H), 3.40–3.72 (m, 1H), 2.15–2.50 (m, 2H), 0.60–2.10 (m, 41H); ^{13}C: δ = 146.65, 124.25, 71.71, 65.75, 56.25, 49.81, 42.65, 42.54, 39.92, 37.91, 37.79, 36.16, 31.75, 28.67, 28.40, 24.69, 24.11, 23.20, 22.96, 21.10, 19.13, 18.64, 12.03. $C_{27}H_{46}O_2$ calcd C 80.5, H 11.5; found C 80.0, H 11.4.

2.8. Synthesis of 7β-hydroxycholesterol 10

To a stirred solution of 7-keto cholesterol **2** (445 mg, 10^{-3} mol) in dry THF (10 mL) was added a 0.4 M solution of $CeCl_3 \cdot H_2O$ in THF/CH_3OH (2/1, 2.5 mL). $NaBH_4$ (38 mg, 10^{-3} mol) was then slowly added and stirring was maintained for 1 h at room temperature. The reaction was quenched by addition of 5% HCl (1 mL) and 7β-hydroxy cholesterol was extracted with CH_2Cl_2. The organic layer was dried over $MgSO_4$, filtered and the solvents were removed in vacuo. The crude product was purified by chromatography on a silicagel column using ethylacetate as eluent affording the expected 7β-hydroxycholesterol **10** in 75% yield.

White solid; mp: 77 °C; ^{1}H NMR: δ = 5.27 (s, 1H), 3.82–3.84 (m, 1H), 3.50–3.57 (m, 1H), 0.67–2.31 (m, 43H); ^{13}C: δ = 144.25, 126.22, 74.13, 72.20, 56.73, 56.24, 59.04, 43.70, 41.68, 40.27, 37.71, 37.21, 36.98, 36.50, 32.35, 29.31, 28.78, 27.15, 24.61, 2..58, 23.32, 21.85, 19.93, 19.55, 12.60. $C_{27}H_{46}O_2$ calcd C 80.5, H 11.5; found C 80.5, H 11.5.

2.9. Synthesis of cholest-4-ene-3,6-diol 11

To a stirred solution of $LiAlH_4$ (210 mg, 5.52×10^{-3} mol) in dry THF (10 mL) was slowly added at 0 °C a solution of cholest-4-ene-3,6-dione 7 (550 mg, 1.38×10^{-3} mol) in dry THF. Stirring was maintained for 24 h allowing the solution

to warm to room temperature. The reaction was quenched by addition of water (397 μL, 2.20×10^{-2} mol). After filtration through a pad of Celite, the organic layer was dried over $MgSO_4$, filtered and the solvents were removed in vacuo. The crude product was purified by chromatography on a silicagel column using petroleum ethylacetate as eluent affording the expected compound **11** in 72% yield.

White solid; mp: 157 °C; ^{1}H NMR: δ = 5.75–6.925 (m, 1H), 5.45–5.2 (m, 1H), 3.7–4.35 (m, 3H), 0.45–2.6 (m, 41H); ^{13}C: δ = 149.61, 120.25, 69.02, 68.33, 56.53, 56.21, 54.61, 42.91, 39.88, 38.31, 36.51, 36.14, 34.74, 28.53, 28.40, 24.22, 23.21, 22.95, 20.08, 19.04, 12.35. $C_{27}H_{46}O_2$ calcd C 80.5, H 11.5; found C 80.3, H 11.2.

2.10. Synthesis of 6β-hydroxy cholestanol 13

To a stirred solution of commercial 6-ketocholestanol 12 (444 mg, 10^{-3} mol) in dry THF (10 mL) was added a 0.4 M solution of $CeCl_3 \cdot H_2O$ in THF/CH_3OH (2/1, 2.5 mL). $NaBH_4$ (38 mg, 10^{-3} mol) was then slowly added and stirring was maintained for 1 h at room temperature. The reaction was quenched by addition of 5% HCl (1 mL) and the product was extracted with CH_2Cl_2. The organic layer was dried over $MgSO_4$, filtered and the solvents were removed in vacuo. The crude product was purified by chromatography on a silicagel column using ethylacetate as eluent affording the expected 6β-hydroxycholestanol **13** in 60% yield.

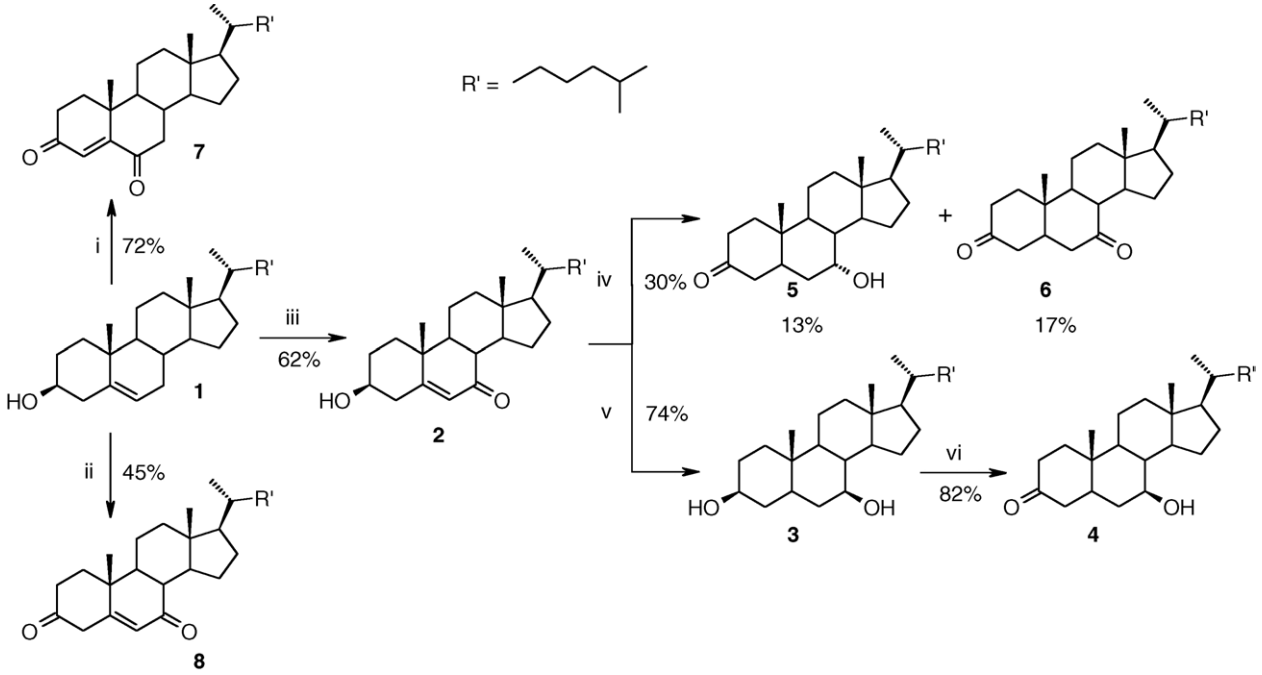
White solid; mp: 168 °C; ^{1}H NMR: δ = 7.25–7.36 (m, 1H), 3.61–3.94 (m, 2H), 0.65–2.36 (m, 45H); ^{13}C: δ = 71.9, 71.3, 57.1, 56.9, 53.9, 47.2, 42.5, 40.6, 39.8, 39.4, 37.2, 36.1, 35.7, 35.4, 31.4, 30.4, 28.7, 27.9, 24.6, 23.5, 23.2,21.1, 19.4, 16.5, 12.1. $C_{27}H_{48}O_2$ calcd C 80.2, H 11.9; found C 80.0, H 11.1.

3. Results and discussion

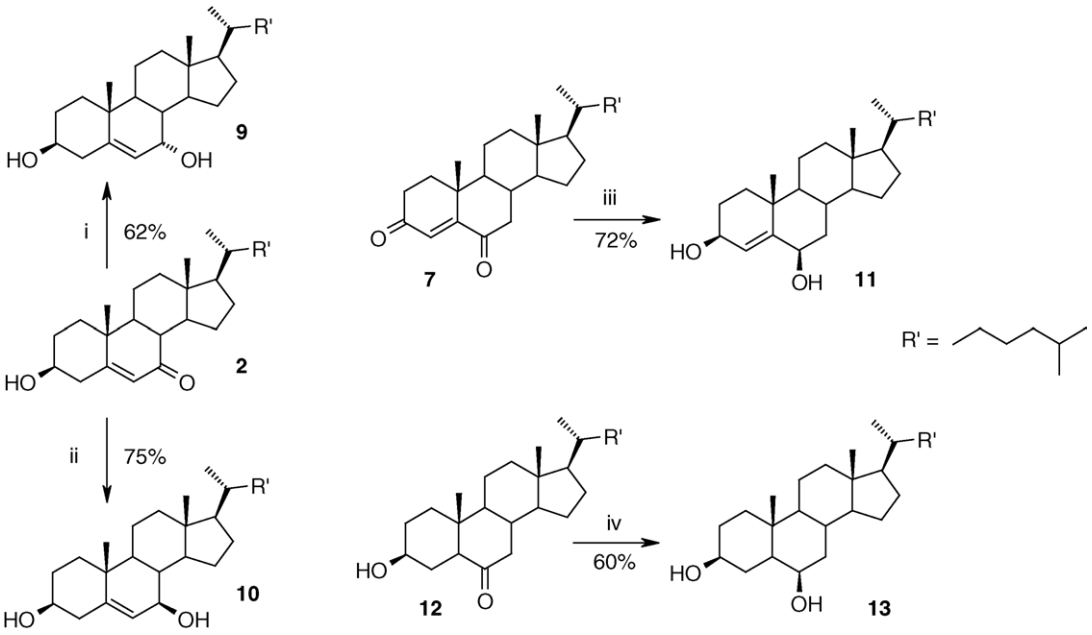
3.1. Chemistry

In the present study, various oxygenated cholesterol derivatives **1**–**13** were prepared from new synthetic methods or literature modified procedures as outlined in Schemes 1 and 2. All these oxygenated derivatives were prepared in only one or two steps in high scale synthesis from commercial and inexpensive reagents. It is noteworthy that all these compounds were previously isolated as byproducts in low chemical yields from numerous oxidation processes but no methods were reported to date for their efficient synthesis.

According to a modified procedure described by Kinney et al. the high scale synthesis of 7-keto cholesterol **2** has been performed from cholesterol **1** in the presence of *N*-hydroxyphtalamide in a continuously aerated EtOAc/acetone (1/1) solution at 50–60 °C for 72 h [23,24]. Subsequent treatment of the crude mixture with $CuCl_2$ in pyridine afforded the expected product **2** as a white solid in 62% yield. 7β-hydroxy cholestanol **3** was easily prepared in 74% isolated yield from derivative **2** by lithium–ammonia reduction at −78 °C. Oxidation of compound **3** in refluxing toluene with silver carbonate on Celite afforded 7β-hydroxy cholestanone **4** in 82% yield. On the other hand, parent compounds 7α-hydroxy cholestanone **5** and 3,7-cholestanedione **6** were



Scheme 1. (i) PCC CH_2Cl_2, 20 °C, 96 h. (ii) Peracetic acid, $C_5H_{10}O_4Cr$, CH_2Cl_2, 20 °C, 48 h. (iii) (a) O_2, acetone/ethylacetate, *N*-hydroxyphtalimide, benzoylperoxide, 50 °C, 72 h; (b) $CuCl_2$, pyridine, 0 °C, 24 h. (iv) (a) L-Selectride, THF, −78 °C, 24 h; (b) Ag_2CO_3-Celite, toluene, 110 °C, 12 h. (v) (a) Li, NH_3, THF, −78 °C, 1 h. (vi) Ag_2CO_3-Celite, toluene, 110 °C, 12 h.



Scheme 2. (i) L-Selectride, THF, −78 °C, 24 h. (ii) $AlLiH_4$, THF, 0 °C, 24 h. (iii) $AlLiH_4$, THF, 20 °C, 24 h. (iv) $NaBH_4$, MeOH, 20 °C, 24 h.

easily synthesized in a one step sequence from derivative **2** by L-Selectride reduction at −78 °C in THF for 5 h and subsequent oxidation with silver carbonate on Celite in refluxing toluene. Purification of the crude residue on a silicagel column afforded the expected products **5** and **6** in respectively 13 and 17% yield. Moreover, cholest-5-ene-3,7-dione **8** was prepared from cholesterol **1** according to a modified procedure reported by Corey et al. involving a cyclic chromate ester/peroxyacetic acid oxidant reagent [25]. 7α and 7β-hydroxy cholesterol **9** and **10** were obtained in 62 and 75% yield involving, respectively, K-Selectride and $NaBH_4/CeCl_3{\cdot}H_2O$ reducing agents. On the other hand, diol compounds **11** and **13** came from the corresponding keto derivatives **7** and **12** according to modified well known procedures.

All these compounds were isolated in satisfactory yields (13–82%) and both chemical structures were consistent with both analytical and spectroscopic data (^1H and ^{13}C NMR).

3.2. Biological investigation

All the synthesized compounds were screened for antimicrobial activity against several yeast strains, Gram-positive and Gram-negative bacteria strains [26]. Six out of eleven oxygenated cholesterol derivatives tested in the present study were found to have no activity against the micro-organisms listed in Table 1.

Results of the remaining five compounds showed that they have some antifungal activities but no antibacterial activities. The best results have been encountered using hydroxy ketones **2**, **4**–**5** showing activities against *S. cerevisiae* (ATCC 28383) at a concentration of 1.5, 0.4, and 0.4 μg/mL, respectively. The presence of a keto group and an hydroxy moiety on the 3,7-cholestane structure positions seems to be necessary to improve the biological activity of the molecule against fungi. Thus, most antifungal drugs are sterol biosynthesis inhibitors (SBIs) acting as site-specific inhibitors at different steps of the ergosterol biosynthesis, the predominant sterol in most fungi [27]. These SBIs compounds **2**, **4** and **5** are mimics of the carbocationic intermediates involved in Δ8–Δ7 sterol isomerase reaction since this process is conducted by the initial addition of a proton to the α face of C-9 giving a stabilized carbonium ion at C-8. This high energy intermediate is converted into Δ7 by removal of the 7β or 7α hydrogen atom. Thus, in our case, these compounds seem to inhibit Δ8–Δ7 isomerase and to arrest cell proliferation. On the other hand, compound **2** has exhibited activities against *Candida albicans* (CIP 1663-86) and Amphotericine B and miconazole resistant strain *C. albicans* (CIP 1180-79) at a concentration of 1.5 μg/mL. In all other cases, moderate results have been

Table 1
Antimicrobial activity of oxygenated cholesterol derivatives **2**–**13**

Sample CIP	Antimicrobial activity (IC_{50}) (μg/mL)			
	S. cerevisiae (ATCC 28383)	*S. aureus* (53-154)	*C. albicans* (1180-79)	*C. albicans* (1663-86)
2	1.5	>50	1.5	1.5
4	0.4	>50	12.5	>50
5	0.4	>50	12.5	>50
6		>50	>50	>50
7	0.8	>50	3.1	25
8	>50	>50	>50	>50
9	>50	>50	>50	>50
10	>50	>50	>25	>50
11	12.5	>50	>25	12.5
12	–	6.2	12.5	>50
13	>25	–	6.2	>25

encountered even if an antibacterial activity against *S. aureus* at a concentration of 6.2 μg/mL has been noticed using compound **12**.

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