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One step synthesis of 6-oxo-cholestan-3β,5α-diol

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ABSTRACT

Cholesterol metabolism has been recently linked to cancer, highlighting the importance of the characterization of new metabolic pathways in the sterol series. One of these pathways is centered on cholesterol-5,6-epoxides (5,6-ECs). 5,6-ECs can either generate dendrogenin A, a tumor suppressor present in healthy mammalian tissues, or the carcinogenic cholestane-3β,5α,6β-triol (CT) and its putative metabolite 6-oxo-cholestan-3β,5α-diol (OCDO) in tumor cells. We are currently investigating the identification of the enzyme involved in OCDO biosynthesis, which would be highly facilitated by the use of commercially unavailable [^{14}C]-cholestane-3β,5α,6β-triol and [^{14}C]-6-oxo-cholestan-3β,5α-diol. In the present study we report the one-step synthesis of [^{14}C]-cholestane-3β,5α,6β-triol and [^{14}C]-6-oxo-cholestan-3β,5α-diol by oxidation of [^{14}C]-cholesterol with iodide metaperiodate (HIO_4).

1. Introduction

Sterols are unique lipids comprised of a rigid tetracyclic core linked to a flexible hydrocarbon side chain and are facile templates for oxygenation to oxysterols, through both enzymatic and non-enzymatic routes. Oxysterols, like steroid hormones, have specific physiological properties and deregulation of their metabolism is associated with several pathologies including cancer [1–6]. Some oxysterol metabolic pathways represent novel targets for the development of anticancer agents [2,5,7]. Working on cholesterol (**1**) metabolism and cancer, our team has recently established the molecular identification of the cholesterol-5,6-epoxide hydrolase (ChEH) [8]. ChEH hydrolyzes 5,6α-epoxy-cholesterol (5,6α-EC (**2a**)) and 5,6β-epoxy-cholesterol (5,6β-EC (**2b**)) into cholestane-3β,5α,6β-triol (CT (**3**)) [9]. We found that 5,6α-EC was enzymatically metabolized into dendrogenin A (5α-hydroxy-6β-[2-(1H-imidazol-4-yl)-ethylamino]-cholestan-3β-ol) in the presence of histamine [4], and dendrogenin A was found to be produced by several mammalian tissues, to display tumor suppressor properties [4,10] and to stimulate neural stem cell differentiation [11]. We showed that important drugs with anticancer and chemopreventive properties like the antiestrogen tamoxifen and natural products such as docosahexaenoic acid inhibit ChEH which led to the accumulation of 5,6-ECs in tumor cells [4,8,9,12–15]. We subsequently reported that the accumulation of 5,6-ECs was responsible for the re-differentiation of cancer cells and the cytotoxic properties of ChEH inhibitors [12,13,15]. On the other hand, these drugs inhibit the production of CT (**3**) and its product of oxidation, 6-oxo-cholestan-3β,5α-diol (OCDO) (**4**) (Fig. 1). **3** was reported to be genotoxic [16], while **4** was reported to display tumor promoting properties [17], to inhibit natural killer cell mediated cytotoxicity [18], to inhibit the formation of E-rosette [19] and the cytotoxic activity of T lymphocytes [20]. It is thus important to study the metabolism of compounds **3** and **4** and to determine whether they are produced through an enzymatic process. This prompted us to develop a simple method for the synthesis of **3** and **4** in their unlabeled and ^{14}C-labeled forms to study and identify the enzyme that catalyzed the transformation of **3** into **4**.

2. Materials and methods

5,6α-EC, 5,6β-EC were synthesized as reported before [8,10]. CT and OCDO were synthesized as reported in the present study. [^{14}C]-cholesterol was supplied by Perkin–Elmer and all other reagents were purchased from Sigma–Aldrich (St. Louis, MO). All commercially available chemicals were used without further purification. The progress of the reactions was monitored by TLC using silica gel aluminum sheets and detection with 50% methanolic H_2SO_4. Atmospheric pressure column chromatography was performed on Merck-grade 7734 70–230 mesh silica and reversed-phase HPLC on a Perkin–Elmer LC200 series with Ultrasep ES 100 RP 18, 6.0 μm; Bischoff, Leonberg, Germany. Melting points

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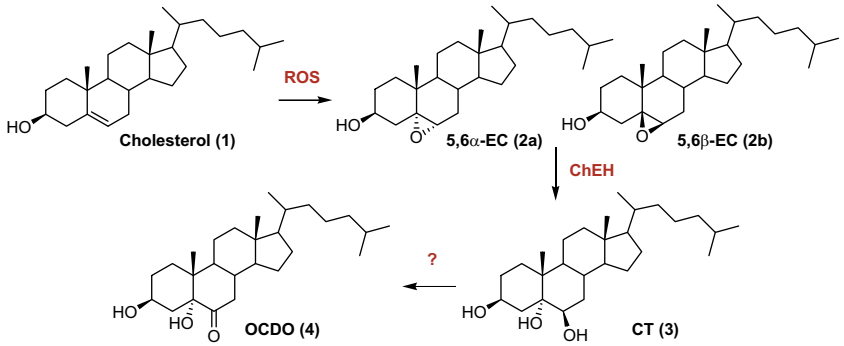

Fig. 1. Oxidation of cholesterol by H_2O_2 can give 5,6-EC (**2a** and **2b**) in mammalian cells. **2a** and **2b** are subsequently metabolized into CT (**3**) by ChEH. **3** is oxidized into OCDO (**4**) by a yet unknown enzyme.

were determined with a Kofler apparatus and are uncorrected. ^1H and ^{13}C NMR spectra were recorded on a Bruker spectrometer AC300. Solutions were prepared in deuterated methanol (CD_3OD), with tetramethylsilane (TMS) as internal standard. EI-MS and CI-MS spectra were obtained with a quadrupolar NERMAG R10-10 spectrometer. Infrared (IR) spectra were recorded on a Perkin–Elmer IR-881 spectrometer. Optical rotations were measured with a PerkinElmer 241 polarimeter. Elemental analyses were carried out at the Institut des Sciences Analytiques, Villeurbanne (France) with the MA-E2–01 method for carbon and hydrogen composition and the MA-E2-13 for oxygen.

2.1. General procedure for the oxidation of cholesterol and oxysterols with HIO_4

Pure THF (15 mL) in aqueous iodide metaperiodate (5.5 mL, 10 mmol, 488 mM, 10 equivalents) was added with stirring to a solution of **1**, **2a**, **2b** or **3** (1 mmol, 48.8 mM). The mixture was stirred at room temperature and turned yellow, orange and brown in a day. The completion of the reaction was shown by TLC. The mixture was washed with excess 10% aqueous sodium thiosulfate and a white precipitate appeared in a colorless solution. The resulting mixture was extracted with ethyl acetate (40 mL). The extract was washed with water, 5% aqueous sodium bicarbonate and brine. The organic layer was then dried over $MgSO_4$, and the solvent removed under reduced pressure. The crude product was purified by column chromatography (0–100% hexane in diethylether and 0–100% diethylether in ethyl acetate) to obtain the product of interest as a white solid.

*2.2. Synthesis of 3β-cholestane-5α,6β-triol (**3**) from cholesterol (**1**)*

The reaction was completed in 26 h with an 85% yield. Rf [EtOAc] = 0.26; mp 240 °C (lit. 239–241 °C [22]); $[\alpha]_D^{20}$ = +2.4° dm^{-1} g^{-1} mL (EtOH) [ref. $[\alpha]_D^{20}$ = +2.4° dm^{-1} g^{-1} mL]; IR (cm^{-1}): 3500–3100, 2939, 2867, 1467, 1375 and 1063; 1H NMR (CD_3OD) ı: 4.03 (m, 1H, H-3),3.47 (t, 1H, H-6), 1.17 (s, 3H, CH3-19), 0.95 (d, 3H, CH3-21), 0.90 (d-d, 6H, CH3-26 & CH3-27), 0.73 (s, 3H, CH3-18); 13C NMR (CD_3OD) 212.08, 80.87, 67.30, 56.37, 56.12, 44.56, 43.12, 42.44, 41.79, 39.59, 39.47, 37.29, 36.46, 36.10, 35.72, 30.38, 29.77, 28.06, 28.00, 23.92, 23.84, 22.81, 22.55, 21.44, 18.63, 14.08, 12.02; (MS (DCI/NH_3) *m/z*: 420 (MH^+), 438 MNH_4^+; Anal. Calcd for $C_{27}H_{48}O_3$: C, 77.09; H, 11.50; O, 11.41; Found: C, 77.22; H, 11.77; O, 11.00.

*2.3. Synthesis of 6-oxo-cholestan-3β,5α-diol (**4**) from cholesterol (**1**)*

The reaction was completed in 24 days with a 30% yield. Rf [EtOAc] = 0.67; mp 232 °C (lit. 231–232 °C [23]); $[\alpha]_D^{20}$ = −35° dm^{-1} g^{-1} mL (EtOH) [ref. $[\alpha]_D^{20}$ = −35° dm^{-1} g^{-1} mL]; IR (cm^{-1}):3500–3100, 2951, 2867, 1701, 1466, and 1375; 1H NMR (CD3OD) ı: 3.92 (m, 1H, H-3), 0.96 (d, 3H, CH_3-21), 0.90 (d-d, 6H, CH_3-26 & CH_3-27), 0.8 (s, 3H, CH_3-19), 0.71 (s, 3H, CH_3-18); NMR δC (75 MHz, DMSO-*d*6) 73.94, 65.77, 62.93, 56.16, 56.10, 55.90, 52.03, 50.43, 44.62, 42.94, 40.09, 38.01, 36.13, 35.73, 32.57, 31.24, 29.67, 28.32, 27.89, 27.33, 23.92, 23.73, 23.16, 22.89, 21.11, 19.92, 18.90, 16.34, 12.62; MS (DCI/NH_3) *m/z*: 418 (MH^+), 436 MNH_4^+; Anal. Calcd for $C_{27}H_{46}O_3$: C, 77.46; H, 11.07; O, 11.46; Found: C, 77.24; H, 10.90; O, 11.86.

*2.4. Synthesis of [^{14}C]-3β-cholestane-5α,6β-triol (**5**) and [^{14}C]-6-oxo-cholestan-3β,5α-diol (**6**) from [^{14}C]-cholesterol*

5 and **6** were synthesized as described above. Briefly, [^{14}C]-cholesterol (20 μci, 0.4 μmol) was dissolved in THF (150 μL), and a solution of aqueous iodide metaperiodate was added (55 μL, 4 μmol). The mixture was stirred at room temperature for 26 h for the production 5 (conditions A) and 24 days to produce **6** (conditions B). At the end of the reaction, the mixture was washed with excess10% aqueous sodium thiosulfate and extracted with ethyl acetate. The extract was washed with water, 5% aqueous sodium bicarbonate and water. The solvent was removed under reduced pressure. Under conditions A, **5** (18 μCi) was obtained with a radiochemical purity >95%. Under conditions B, the extract was purified isocratically by reversed-phase HPLC (Ultrasep ES 100 RP 18, 6.0 μm; Bischoff, Leonberg, Germany) with MeOH/H_2O (95:5) as eluant at a flow rate of 0.7 ml/min. We obtained 6 (7 μCi), with a >98% radiochemical purity.

*2.5. Metabolism of [^{14}C]-3β-cholestane-5α,6β-triol (**5**) by MCF-7 cells*

MCF-7 cells were from the American Type Culture Collection (ATCC) and cultured until passage 30. MCF-7 were grown in RPMI 1640 medium supplemented with 5% fetal bovine serum (FBS), penicillin and streptomycin (50 U/ml) in a humidified atmosphere with 5% CO_2 at 37 °C. MCF-7 cells were seeded in 100 mm plates at 1×10^5, then incubated with [^{14}C]-5 (final concentration 0.6 μM; 20 μCi/μmol) in the presence of solvent vehicle (EtOH 0.1%) for 8 h. The cells were scraped and pelleted by centrifugation for 10 min at 1500 rpm, and then extracted as described above. Samples were spotted onto Fluka 20 × 20 silica gel plates previously heated for 1 h at 100 °C and developed using EtOAc. The radioactive metabolites were identified on TLC plates by co-migration with authentic standards by autoradiography using Kodak Biomax

MS film (Sigma–Aldrich). The Rf for CT (**3** and **5**) and OCDO (**4** and **6**) were 0.26 and 0.67 respectively.

3. Results and discussion

In the search for a simple and direct method to produce **3** and **4**, we chose to study the oxidation of cholesterol by HIO_4 (Table 1). **1** is oxidized into **3** at room temperature using **3** equivalents of HIO_4, while no **4** was found even after up to 20 days incubation, consistent with the Graber et al's report [21]. They found that the reaction could occur only for 3β-acetyl-cholesterol which adds waste generation acetylation/deacetylation steps that should be avoided in the context of ^{14}C-labelled compounds. Increasing the HIO_4 concentration to 10 equivalents improved the yield to 85% at 1 day and gave **4** at 32% yield after 20 days. The monitoring of the progress of the reaction by TLC showed the appearance of 5,6-ECs which are consumed during the reaction. Under the same conditions, 10 equivalents of HIO_4 in the presence of **2a** and **2b** gave both **3** at an 88% yield at 1 day and **4** at a 27% yield at day 20. Finally the oxidation of **3** by 10 equivalents of HIO_4 gave **4** at a 45% yield. Increasing the temperature decreased the yield of the reaction, changing the concentration of HIO_4 does not improve the yield of the reaction, replacing HIO_4 by $HClO_4$ gave no reaction, establishing a specific action of HIO_4 as a catalyst to the production of **3** an **4** from **1** (Table 1). These data established that the synthesis of **3** and **4** is possible from **1** in one step with a good yield.

Based on these data, we have done the same experiments with [^{14}C]-cholesterol using 10 equivalents of HIO_4. 1 day of incubation of [^{14}C]-cholesterol with HIO_4 gave [^{14}C]-CT (**5**) with a 80% yield with a radio-purity greater than 98% after HPLC purification. The iodide metaperiodate oxidation of [^{14}C]-cholesterol for 20 days gave [^{14}C]-OCDO (**6**) at a 30% yield with a radio-purity greater than 98% after HPLC purification. These data established that we succeeded in the synthesis of **5** and **6** in one step with a good yield.

As an illustration of the use of **5** and **6**, in Fig. 2 we show that the incubation of breast cancer cells with 5 gave a transformation product that co-migrated with 6 on TLC. This established that the metabolism of **5** and **6** can be carried out with a small number of cells using these labelled compounds. The use of **5** and **6** has enabled us to identify the enzymes involved in the biosynthesis of **6** from **5** and in the biosynthesis of **5** from **6** (Voisin et al., manuscript in preparation). The same synthesis was used successfully to get deuterated CT and OCDO from deuterated cholesterol (data not shown).

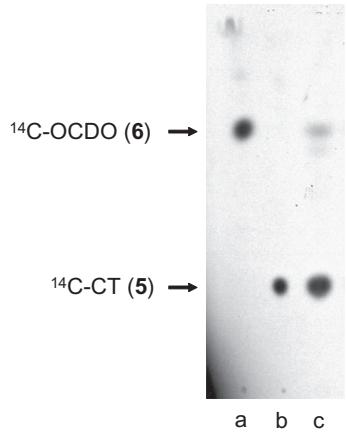

Fig. 2. Metabolism of **5** in MCF-7 cells. (A) Synthetic compound **5**, (B) synthetic compound **6**, (C) extract of cells incubated in the presence of compound **5** gave compound **6**. 100,000 MCF-7 cells were incubated for 8 h in the presence of compound **5**. Cells were washed and lipids were extracted as described in Section 2. Sterols were separated by thin layer chromatography (TLC) and the TLC was revealed by autoradiography.

In a previous study Graber et al. reported that 3β-acetylated-**3** did not afford 3β-acetylated-**4** using 3 equivalents of HIO_4 [21]. Our data show that using 10 equivalents of HIO_4, free cholesterol (non acetylated) can give both **3** and **4** in good yields through the intermediate of **2**, establishing that a one step synthesis of 5 was possible. We found that compounds **1**, **2a**, **2b** and **3** react with HIO_4 to give **4** with the same yield. The fact that **3** can give **4** established that **3** is the probable intermediate after compounds **2a** and **2b** in the oxidation of **1** into **4** following a route of chemical transformation as shown in Fig. 3.

The iodide metaperiodate oxidation of [^{14}C]-cholesterol is a simple and direct way to obtain **5** and **6** in good yield.

In conclusion, a one step synthesis of cholestane-3β,5α,6β-triol **2** and 6-oxo-cholestan-3β,5α-diol **3** from cholesterol is presented and constitutes an easy synthesis of [^{14}C]-cholestane-3β,5α,6β-triol (**5**) and [^{14}C]-6-oxo-cholestan-3β,5α-diol (**6**) from [^{14}C]-cholesterol.

Table 1
Synthesis of CT (**3**) and OCDO (**4**) from cholesterol (**1**), 5,6α-EC (**2a**), 5,6β-EC (**2b**) and CT (**3**) in various conditions. r.t.: room temperature.

Sterol	Catalist	Equivalent	Temperature	Product	Incubation time (day)	Yield %
1	HIO_4	3	r.t.	3	1	12
				4	20	0
			Reflux	3	1	0
				4	1	0
		10	r.t.	3	1	85
				4	20	32
			Reflux	3	1	0
				4	1	0
		20	r.t.	3	1	6
2a		10	r.t.	3	1	88
				4	20	27
2b		10	r.t.	3	1	88
				4	20	27
3		10	r.t.	4	20	45
				4	20	0
1	$HClO_4$	3	r.t.	3	1	1
				4	20	0
			Reflux	3	1	1
				4	1	0
		10	r.t.	3	1	0
				4	20	0

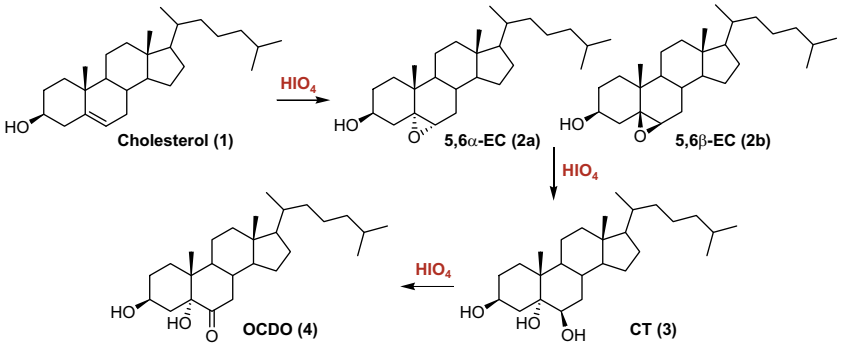

Fig. 3. Chemical synthesis of **3** and **4** by HIO_4 from **1**.

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