

## Oxidized Derivatives of Dihydrobrassicasterol: Cytotoxic and Apoptotic Potential in U937 and HepG2 Cells

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**ABSTRACT:** The ability of phytosterol compounds to reduce plasma serum cholesterol levels in humans is well investigated. However, phytosterols are structurally similar to cholesterol with a double bond at the C<sub>5-6</sub> position and are therefore susceptible to oxidation. Much research has been carried out on the biological effects of cholesterol oxidation products (COPs) in vitro. In contrast, there is less known about phytosterol oxidation products (POPs). From previous studies, it is apparent that oxidized derivatives of the phytosterols,  $\beta$ -sitosterol and stigmasterol, are cytotoxic in vitro but are less potent than their COP counterparts. In the present study, the cytotoxic and apoptotic potential of oxidized derivatives of dihydrobrassicasterol (DHB) including 5 $\alpha$ ,6 $\alpha$ -epoxyergostan-3 $\beta$ -ol ( $\alpha$ -epoxide), 5 $\beta$ ,6 $\beta$ -epoxyergostan-3 $\beta$ -ol ( $\beta$ -epoxide), ergost-5-en-7-on-3 $\beta$ -ol (7-keto), ergost-5-ene-3 $\beta$ ,7 $\beta$ -diol (7- $\beta$ -OH), and ergostane-3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -triol (triol) were evaluated in the U937 and HepG2 cell lines. In general, 7-keto, 7- $\beta$ -OH, and triol derivatives had a significant cytotoxic impact on U937 and HepG2 cells. The oxides appear to be more toxic toward U937 cells. In line with previous findings, the POPs investigated in this study were less potent than the equivalent COPs. The results add to the body of data on the toxicity of individual POPs.

**KEYWORDS:** U937, HepG2, dihydrobrassicasterol oxidation products, cytotoxicity, apoptosis

### ■ INTRODUCTION

Plant sterols or phytosterols are present in the unsaponifiable matter of vegetable oils and fats. Phytosterols have been incorporated into a range of cholesterol-lowering products, and inclusion of these products in the diet has been documented to reduce serum cholesterol levels in human clinical trials.<sup>1</sup> For this reason their addition to foods has increased significantly over the past decade. Phytosterols have an unsaturated ring structure similar to cholesterol (Figure 1). Due to the presence of a double bond between C5 and C6, sterols can undergo oxidative processes. Therefore, cholesterol and phytosterols present in foods are susceptible to oxidation especially in those foods which have been exposed to heat treatments in the presence of oxygen or have been stored for long periods subjected to sunlight and oxygen. Oxidation causes the formation of cholesterol oxidation products (COPs) and phytosterol oxidation products (POPs).<sup>2</sup> It has been reported that POPs are present in phytosterol-enriched products at concentrations of approximately 0.1% (12–68  $\mu$ g/g).<sup>3,4</sup> The recommended daily intake of phytosterol-enriched spreads is four servings of 12 g each which could potentially equate to a POP intake of 0.6–3.4 mg.

The content and profile of phytosterols/stanols added to foods is determined by the ingredient source.<sup>5</sup> In tall oil products,  $\beta$ -sitosterol predominates, followed by sitostanol and campesterol. In soybean products,  $\beta$ -sitosterol, stigmasterol, and campesterol are the major components. Vegetable oils contain mainly  $\beta$ -sitosterol but also contain sitostanol and campesterol. Garcia-Llatas et al. have reviewed studies investigating POPs in plant sterol-enriched food, and it is evident that  $\beta$ -sitosterol oxides predominate.<sup>6</sup> In general, 7-keto and 7-OH oxide derivatives of all phytosterols are the most

abundant followed by the epoxide derivatives. To date, it has been difficult to separate DHB from campesterol and therefore accurately quantify DHB oxides. Thus, the contribution of DHB oxides to the diet is unclear but is anticipated to be low in comparison to POPs generated from the major phytosterol,  $\beta$ -sitosterol. However, the potential effects of DHB oxides may be of more significance than  $\beta$ -sitosterol oxides as they are closer in structure to the parent cholesterol oxides.

In addition to dietary intake, POPs in plasma may be derived from phytosterols penetrating the skin via cosmetic products and subsequently oxidized by UV light.<sup>7</sup> The majority of research on the biological effects of sterol oxidation products has focused on COPs.<sup>8–10</sup> In relation to POPs, proposed biological effects include modulation of cholesterol metabolism via liver X receptor (LXR), lipid lowering and antidiabetic properties, modulation of oxidation capacity of cytochrome P450, cytotoxicity in vitro and in vivo, modulation of inflammation and immunity, and estrogenic and/or androgenic activity.<sup>11</sup> However, definitive information on the potential toxic effects of POPs is scarce mainly due to the lack of commercial availability of the individual oxide standards. Existing knowledge on the cytotoxicity of POPs in vitro generally pertains to blends of POPs rather than pure compounds. There is some evidence that mixes of sterol oxidation compounds act in a different way to a single purified oxide compound.<sup>12</sup>

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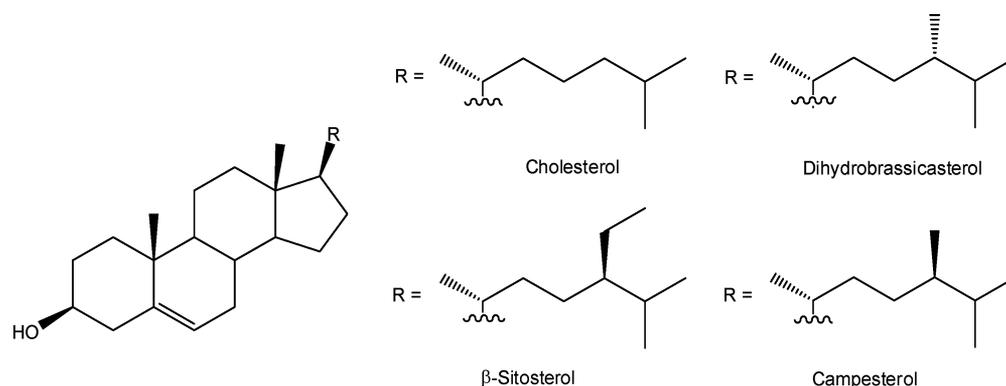


Figure 1. Structures of cholesterol and common phytosterols.

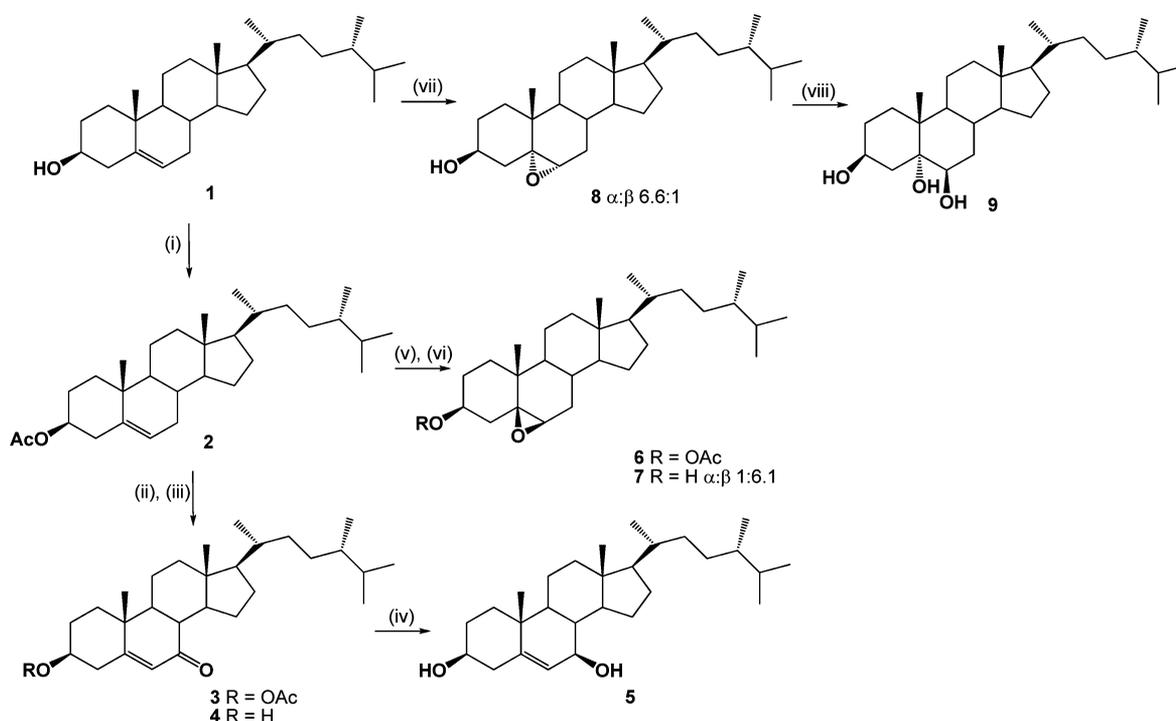


Figure 2. Structures of the DHB oxides investigated in the present study. (1) DHB; (2) DHB acetate; (3) ergost-5-en-7-on-3 $\beta$ -ol acetate, 7-keto acetate; (4) ergost-5-en-7-on-3 $\beta$ -ol, 7-keto; (5) ergost-5-ene-3 $\beta$ ,7 $\beta$ -diol, 7- $\beta$ -OH; (6) 5 $\beta$ ,6 $\beta$ -epoxyergostan-3 $\beta$ -ol acetate,  $\beta$ -epoxide acetate; (7) 5 $\beta$ ,6 $\beta$ -epoxyergostan-3 $\beta$ -ol,  $\beta$ -epoxide; (8) 5 $\alpha$ ,6 $\alpha$ -epoxyergostan-3 $\beta$ -ol,  $\alpha$ -epoxide; (9) ergostane-3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -triol, triol. (i) Ac<sub>2</sub>O, pyridine (87%); (ii) CrO<sub>3</sub>, dimethylpyrazole, CH<sub>2</sub>Cl<sub>2</sub> -20 to 5 °C (43%); (iii) K<sub>2</sub>CO<sub>3</sub>, MeOH, H<sub>2</sub>O (95%); (iv) CeCl<sub>3</sub>·7H<sub>2</sub>O, NaBH<sub>4</sub>, MeOH, (71%); (v) KMnO<sub>4</sub>-CuSO<sub>4</sub>·5H<sub>2</sub>O, *t*-BuOH, H<sub>2</sub>O, CH<sub>2</sub>Cl<sub>2</sub> (50%); (vi) Na<sub>2</sub>CO<sub>3</sub>, MeOH (83%); (vii) *m*CPBA, CH<sub>2</sub>Cl<sub>2</sub> (89%); (viii) H<sub>2</sub>SO<sub>4</sub>, THF-H<sub>2</sub>O, (74%).

Thus, in order to obtain a more comprehensive insight into the cytotoxicity of POPs, synthesis and evaluation of single oxides is paramount. To date, studies on the effects of individual POPs have involved those derived from  $\beta$ -sitosterol and stigmasterol.<sup>12–17</sup> In general, the oxides of  $\beta$ -sitosterol were similar to the equivalent COPs, however higher concentrations of POPs were required to elicit comparable toxic effects.<sup>6,14–16</sup> In relation to stigmasterol oxides, the 7- $\beta$ -OH, epoxydiol, and diepoxide derivatives were cytotoxic to the U937 cell line and all three induce significant apoptotic cell death.<sup>17</sup> In fact, the diepoxide and epoxydiol derivatives of stigmasterol, which are oxidized on the side chain, were found to be the most cytotoxic of all the derivatives tested in the U937 cell line and are also significantly cytotoxic in HepG2 and Caco-2 cells.<sup>17,18</sup> Stigmasterol differs structurally from cholesterol and  $\beta$ -sitosterol, in that it contains an additional double bond, positioned at C22–23. The presence of the additional double

bond allows for the formation of oxides such as diepoxide and epoxydiol, compounds that do not have cholesterol or  $\beta$ -sitosterol equivalents.<sup>17</sup> Given these novel findings, it is of interest to examine other individual oxides derived from different phytosterols.

In the present study, we report on the synthesis and toxicity of a range of individual oxides of DHB (1) (Figure 2). DHB (1) is a 24-epimer of campesterol. At the C24 position, the methyl group is on the alpha-face in DHB and the beta-face in campesterol (Figure 1). DHB occurs naturally in specific *Chlorella* species<sup>19</sup> and can be biosynthesized from ergosterol.<sup>20</sup> The significance of this key phytosterol becomes clear when one considers that DHB and campesterol are usually quoted as a “campesterol fraction” in analysis of phytosterols in foods (primarily due to the fact that the analysis is done by GC or <sup>1</sup>H NMR and these isomers are only separated by chiral GC or <sup>13</sup>C NMR) and that this campesterol fraction is made up of 2:1

campesterol:DHB.<sup>21</sup> Therefore, DHB content of phytosterol sources can be as high as 10%. The significance of the novel DHB oxides targeted in this study can be related to their potential to answer a fundamental question at the heart of dietary phytosterol supplementation. Once these benchmark compounds have been fully characterized via biological evaluation, key toxic POPs can be identified and hence avoided by assiduous use of phytosterol mixtures in formulation or storage regimens.

Thus, the objective of this study was to synthesize DHB and characterize a range of oxidized derivatives of DHB including 5 $\alpha$ ,6 $\alpha$ -epoxyergostan-3 $\beta$ -ol ( $\alpha$ -epoxide, **8**), 5 $\beta$ ,6 $\beta$ -epoxyergostan-3 $\beta$ -ol ( $\beta$ -epoxide, **7**), ergost-5-en-7-on-3 $\beta$ -ol (7-keto, **4**), ergost-5-ene-3 $\beta$ ,7 $\beta$ -diol (7- $\beta$ -OH, **5**), and ergostane-3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -triol (triol, **9**) (Figure 2). The U937 cell line is commonly used as a macrophage reference model in studies investigating the cytotoxic effects of COPs and POPs.<sup>15,22</sup> Previous studies have demonstrated that the COPs are cytotoxic but do not induce apoptosis in HepG2 cells.<sup>23</sup> Thus, in this study the cytotoxicity and apoptotic potential of each oxidized derivative was assessed in the U937, monocytic blood cell line and the HepG2, hepatic cell line.

## MATERIALS AND METHODS

**Materials.** Cell lines were obtained from the European Collection of Animal Cell Cultures (Sailsbury, U.K.).

**Chemicals.** All chemicals were obtained from Sigma Chemical Co. (Poole, U.K.) unless otherwise stated. Information on the purity of COPs was obtained from Sigma

**Cell Maintenance.** Human monocytic U937 cells were grown in suspension in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS). Human hepatoma HepG2 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) FBS and 1% nonessential amino acids. The cells were grown at 37 °C and 5% (v/v) CO<sub>2</sub> in a humidified incubator. Cells were cultured in the absence of antibiotics. Exponentially growing cells were used in all experiments.

**Description of Synthesis of DHB (1).** Synthetic DHB was obtained in >95% purity (with the remaining <5% being campesterol) via a recently described method.<sup>24</sup> Briefly, DHB (**1**) was synthesized in 6 steps starting from stigmasterol (>95% purity). Stigmasterol was protected via tosylate formation, solvolyzed with methanol, and cleaved under ozonolysis conditions to yield an aldehyde. This aldehyde was converted via Wittig reaction to the required alkene for DHB, which could then be hydrogenated and deprotected to yield DHB in >95% purity (as assigned by <sup>1</sup>H and <sup>13</sup>C NMR). All the DHB oxides described in this manuscript were synthesized from this material.

**Synthesis of POPs: General Procedures.** Melting points were measured on a Uni-Melt Thomas-Hoover capillary melting point apparatus and are uncorrected. Low resolution mass spectra were recorded on a Waters Micromass Quattro Micro mass spectrometer (instrument number QAA1202) in electrospray ionization (ESI) positive and negative modes and a Waters Micromass LCT Premier (instrument number KD160) was used for high resolution acquisitions. Infrared (IR) spectra were recorded as potassium bromide (KBr) disks on a Perkin-Elmer FT-IR Paragon 1000 or a Spectrum One FT-IR spectrophotometer. <sup>1</sup>H NMR (300 MHz) and <sup>13</sup>C NMR (75 MHz) were recorded on a Bruker Avance 300 NMR spectrometer unless otherwise stated. All spectra were recorded at 20 °C in deuterated chloroform (CDCl<sub>3</sub>) with tetramethylsilane (TMS) as an internal standard unless otherwise stated. Chemical shifts ( $\delta_{\text{H}}$  and  $\delta_{\text{C}}$ ) are reported in parts per million (ppm), relative to TMS, and coupling constants are expressed in hertz (Hz). Splitting patterns in <sup>1</sup>H NMR spectra are designated as s (singlet), br s (broad singlet), d (doublet), dd (doublet of doublets), ddd (doublet of doublet of doublets), dt (doublet of triplets), t (triplet), q (quartet), and m

(multiplet). Thin layer chromatography (TLC) was carried out on precoated silica gel plates (Merck 60 PF<sub>254</sub>). Visualization was achieved by UV light (254 nm), vanillin or potassium permanganate staining. Column chromatography was carried out using Kieselgel 60, 0.040–0.063 nm (Merck). Specific rotations were recorded on a Perkin-Elmer 341 polarimeter at 20 °C in the solvents indicated. The sodium D line (589 nm) was used. Abbreviations used: mCPBA, 3-chloroperbenzoic acid; 4-DMAP, 4-(dimethylamino)pyridine; DMSO, dimethyl sulfoxide.

**DHB Acetate (2).** Acetic anhydride (1.037 mL, 11.00 mM) and pyridine (0.885 mL, 11.00 mM) were combined, and DHB (**1**) (2.200 g, 5.50 mM) in CH<sub>2</sub>Cl<sub>2</sub> (50 mL) was added slowly via addition funnel. The reaction mixture was refluxed for 24 h. The reaction mixture was stirred with a saturated aqueous solution of NaHCO<sub>3</sub> (40 mL) for 30 min. The organic layer was extracted with CH<sub>2</sub>Cl<sub>2</sub> (3  $\times$  30 mL). The combined organic layers were washed with 2 M HCl (2  $\times$  30 mL), water (30 mL), and brine (10 mL), dried, filtered, and concentrated under reduced pressure to give a white solid. Purification by flash chromatography on silica gel [hexane:ethyl acetate (85:15)] yielded the title compound (**2**) as a white solid (2.119 g, 87%): mp 135–137 °C; [ $\alpha_{\text{D}}^{20}$  –55.45° (*c* 1.000 in CHCl<sub>3</sub>);  $\nu_{\text{max}}$ (KBr)/cm<sup>-1</sup> 2961, 2938, 2905, 2822, 1731, 1466, 1441, 1367;  $\delta_{\text{H}}$  (300 MHz, CDCl<sub>3</sub>) 0.68 (3H, s, 18-CH<sub>3</sub>), 0.77–2.00 [40H, m containing 0.77–0.93 (12H, overlapping 4  $\times$  d, J 6.8, 21-CH<sub>3</sub>, 26-CH<sub>3</sub>, 27-CH<sub>3</sub> and 28-CH<sub>3</sub>), 1.02 (3H, s, 19-CH<sub>3</sub>), 2.03 (3H, s, COCH<sub>3</sub>), 2.32 (2H, bd, J 7.8), 4.55–4.66 (1H, m, 3 $\alpha$ -H), 5.37 (1H, bd, J 4.2, 6-H);  $\delta_{\text{C}}$  (75 MHz, CDCl<sub>3</sub>) 11.9 (CH<sub>3</sub>), 15.5 (CH<sub>3</sub>), 17.6 (CH<sub>3</sub>), 18.9 (CH<sub>3</sub>), 19.3 (CH<sub>3</sub>), 20.5 (CH<sub>3</sub>), 21.1 (CH<sub>2</sub>), 21.4 (CH<sub>3</sub>), 24.3 (CH<sub>2</sub>), 27.8 (CH<sub>2</sub>), 28.2 (CH<sub>2</sub>), 30.6 (CH<sub>2</sub>), 31.5 (CH), 31.9 (CH), 31.92 (CH<sub>2</sub>), 33.7 (CH<sub>2</sub>), 36.2 (CH), 36.6 (quaternary C), 37.0 (CH<sub>2</sub>), 38.1 (CH<sub>2</sub>), 39.1 (CH), 39.7 (CH<sub>2</sub>), 42.3 (quaternary C), 50.1 (CH), 56.0 (CH), 56.7 (CH), 74.0 (CH), 122.7 (CH), 139.7 (quaternary C), 170.5 (C=O); *m/z* (ESI<sup>+</sup>) 383 [M + H – AcOH]<sup>+</sup> (20%), 149 (50), 116 (100); HRMS calc for C<sub>28</sub>H<sub>47</sub> [M + H – AcOH]<sup>+</sup> 383.3678, found 383.3676. The following peaks were distinguishable for the minor campesterol component at a level <5%: 15.4 (CH<sub>3</sub>), 18.3 (CH<sub>3</sub>), 18.7 (CH<sub>3</sub>), 20.2 (CH<sub>3</sub>), 30.3 (CH<sub>2</sub>), 32.4 (CH), 35.9 (CH), 38.9 (CH), 56.1 (CH).

**Ergost-5-en-7-on-3 $\beta$ -ol Acetate (7-Keto Acetate) (3).** Chromium trioxide (4.751 g, 47.55 mM) was suspended in dry dichloromethane (150 mL) and stirred for 30 min at –25 °C. Dimethylpyrazole (4.571 g, 47.55 mM) was added in one portion and the reaction mixture stirred for 30 min at –20 °C. DHB acetate (**2**) (1.400 g, 3.17 mM) in CH<sub>2</sub>Cl<sub>2</sub> (50 mL) was added and the mixture stirred at –20 °C allowing it to warm to 5 °C over 2.5 h. Ethyl acetate (500 mL) was then added and the brown suspension filtered through Celite. The filtrate was concentrated under reduced pressure to give a brown residue. This residue was purified by chromatography on silica gel using hexane–ethyl acetate (95:5), yielding 7-keto acetate (**3**) (0.620 g, 43%) as a white solid: mp 179–181 °C; [ $\alpha_{\text{D}}^{20}$  –105.40° (*c* 1.000 in CHCl<sub>3</sub>);  $\nu_{\text{max}}$ (KBr)/cm<sup>-1</sup> 2957, 2871, 1729, 1672, 1466, 1376;  $\delta_{\text{H}}$  (300 MHz, CDCl<sub>3</sub>) 0.68 (3H, s, 18-CH<sub>3</sub>), 0.77–2.03 [36H, m, containing 0.77–0.94 (12H, overlapping 4  $\times$  d, J 6.8, 21-CH<sub>3</sub>, 26-CH<sub>3</sub>, 27-CH<sub>3</sub> and 28-CH<sub>3</sub>), 1.21 (3H, s, 19-CH<sub>3</sub>), 2.05 (3H, s, COCH<sub>3</sub>), 2.23 (1H, t, J 10.6), 2.35–2.59 (3H, m), 4.66–4.77 (1H, m, 3 $\alpha$ -H), 5.70 (1H, bd, J 1.5, 6-H);  $\delta_{\text{C}}$  (75 MHz, CDCl<sub>3</sub>) 12.0 (CH<sub>3</sub>), 15.5 (CH<sub>3</sub>), 17.3 (CH<sub>3</sub>), 17.6 (CH<sub>3</sub>), 19.1 (CH<sub>3</sub>), 20.5 (CH<sub>3</sub>), 21.2 (CH<sub>2</sub>), 21.3 (CH<sub>3</sub>), 26.3 (CH<sub>2</sub>), 27.4 (CH<sub>2</sub>), 28.5 (CH<sub>2</sub>), 30.6 (CH<sub>2</sub>), 31.5 (CH), 33.7 (CH<sub>2</sub>), 36.0 (CH<sub>2</sub>), 36.1 (CH), 37.8 (CH<sub>2</sub>), 38.3 (quaternary C), 38.7 (CH<sub>2</sub>), 39.1 (CH), 43.1 (quaternary C), 45.4 (CH), 49.8 (CH), 49.9 (CH), 54.6 (CH), 72.2 (CH), 126.7 (CH), 163.8 (quaternary C), 170.3 (C=O), 201.9 (C=O); *m/z* (ESI<sup>+</sup>) 457 [M + H]<sup>+</sup> (3%), 397 (35), 149 (40), 116 (100); HRMS calc for C<sub>30</sub>H<sub>48</sub>O<sub>3</sub> [M + H]<sup>+</sup> 457.3682, found 457.3693. The following peaks were distinguishable for the minor campesterol component at a level <5%: 15.4 (CH<sub>3</sub>), 18.3 (CH<sub>3</sub>), 18.9 (CH<sub>3</sub>), 20.2 (CH<sub>3</sub>), 28.6 (CH<sub>2</sub>), 30.3 (CH<sub>2</sub>), 32.4 (CH), 35.8 (CH), 38.8 (CH), 54.8 (CH).

**Ergost-5-en-7-on-3 $\beta$ -ol (7-Keto) (4).** A suspension of 7-keto acetate (**3**) (0.550 g, 1.21 mM) in methanol (75 mL) was stirred at rt for 5 min. Potassium carbonate (0.183 g, 1.33 mM) in water (15 mL) was added to the suspension, and the mixture was stirred at rt for 24 h.

The reaction mixture was then partitioned between water (200 mL) and ethyl acetate (100 mL). The organic layer was washed with water (2 × 200 mL) and saturated aqueous sodium chloride (2 × 200 mL) and dried over magnesium sulfate, and the solution was then concentrated under reduced pressure to yield title compound (4) as a white solid (0.476 g, 95%): mp 152–154 °C;  $[\alpha]_D^{20}$  –96.15° (*c* 1.000 in CHCl<sub>3</sub>);  $\nu_{\max}$ (KBr)/cm<sup>-1</sup> 3526, 3338, 2955, 2869, 1673, 1655, 1462, 1375;  $\delta_H$  (300 MHz, CDCl<sub>3</sub>) 0.68 (3H, s, 18-CH<sub>3</sub>), 0.77–2.05 [37H, m, containing 0.77–0.94 (12H, overlapping 4 × d, *J* 6.8, 21-CH<sub>3</sub>, 26-CH<sub>3</sub>, 27-CH<sub>3</sub> and 28-CH<sub>3</sub>), 1.20 (3H, s, 19-CH<sub>3</sub>), 2.24 (1H, t, *J* 11.2), 2.36–2.54 (3H, m), 3.62–3.71 (1H, m, 3 $\alpha$ -H), 5.69 (1H, s, 6-H);  $\delta_C$  (75 MHz, CDCl<sub>3</sub>) 12.0 (CH<sub>3</sub>), 15.5 (CH<sub>3</sub>), 17.3 (CH<sub>3</sub>), 17.6 (CH<sub>3</sub>), 19.1 (CH<sub>3</sub>), 20.5 (CH<sub>3</sub>), 21.2 (CH<sub>2</sub>), 26.3 (CH<sub>2</sub>), 28.5 (CH<sub>2</sub>), 30.6 (CH<sub>2</sub>), 31.1 (CH<sub>2</sub>), 31.5 (CH), 33.8 (CH<sub>2</sub>), 36.1 (CH), 36.4 (CH<sub>2</sub>), 38.3 (quaternary C), 38.7 (CH<sub>2</sub>), 39.1 (CH), 41.8 (CH<sub>2</sub>), 43.1 (quaternary C), 45.4 (CH), 49.9 (2 × CH), 54.6 (CH), 70.4 (CH), 126.0 (CH), 165.6 (quaternary C), 202.6 (C=O); *m/z* (ESI<sup>+</sup>) 415 [M + H]<sup>+</sup> (60%), 149 (40), 116 (100); HRMS calc for C<sub>28</sub>H<sub>47</sub>O<sub>2</sub> [M + H]<sup>+</sup> 415.3576, found 415.3575. The following peaks were distinguishable for the minor campesterol component at a level <5%: 15.4 (CH<sub>3</sub>), 18.3 (CH<sub>3</sub>), 18.9 (CH<sub>3</sub>), 20.2 (CH<sub>3</sub>), 28.6 (CH<sub>2</sub>), 30.3 (CH<sub>2</sub>), 32.4 (CH), 35.8 (CH), 38.8 (CH), 54.8 (CH).

**Ergost-5-ene-3 $\beta$ ,7 $\beta$ -diol (7- $\beta$ -OH) (5).** A suspension of 7-keto (4) (0.340 g, 0.82 mM) and cerium chloride heptahydrate (0.459 g, 1.23 mM) in methanol (15 mL) was stirred at rt for 10 min. Sodium borohydride (0.034 g, 0.90 mM) was added to the suspension and the mixture stirred at rt for 24 h. The reaction was worked up by partitioning between water (50 mL) and ethyl acetate (50 mL). The organic layer was washed with water (2 × 20 mL) and saturated aqueous sodium chloride (2 × 20 mL) and dried over magnesium sulfate, and the solution was then concentrated under reduced pressure to yield the crude product as a white solid. This was purified by chromatography on silica gel eluting with 60% ethyl acetate–hexane, yielding a white solid (5) (0.244 g, 71%): mp 164–166 °C;  $[\alpha]_D^{20}$  –10.20° (*c* 1.000 in CHCl<sub>3</sub>);  $\nu_{\max}$ (KBr)/cm<sup>-1</sup> 3381, 2957, 2869, 1670, 1465, 1383;  $\delta_H$  (300 MHz, CDCl<sub>3</sub>) 0.69 (3H, s, 18-CH<sub>3</sub>), 0.77–1.63 [35H, m, containing 0.77–0.94 (12H, overlapping 4 × d, *J* 6.8, 21-CH<sub>3</sub>, 26-CH<sub>3</sub>, 27-CH<sub>3</sub> and 28-CH<sub>3</sub>), 1.05 (3H, s, 19-CH<sub>3</sub>), 1.76–1.93 (4H, m), 2.02 (1H, dt, *J* 12.6, 3.3), 2.20–2.36 (2H, m), 3.48–3.59 (1H, m, 3 $\alpha$ -H), 3.84 (1H, d, *J* 7.8, 7-H), 5.28 (1H, s, 6-H);  $\delta_C$  (75 MHz, CDCl<sub>3</sub>) 11.9 (CH<sub>3</sub>), 15.5 (CH<sub>3</sub>), 17.6 (CH<sub>3</sub>), 19.0 (CH<sub>3</sub>), 19.2 (CH<sub>3</sub>), 20.6 (CH<sub>3</sub>), 21.1 (CH<sub>2</sub>), 26.4 (CH<sub>2</sub>), 28.5 (CH<sub>2</sub>), 30.6 (CH<sub>2</sub>), 31.47 (CH), 31.53 (CH<sub>2</sub>), 33.8 (CH<sub>2</sub>), 36.2 (CH), 36.5 (quaternary C), 37.0 (CH<sub>2</sub>), 39.1 (CH), 39.6 (CH<sub>2</sub>), 40.9 (CH), 41.7 (CH<sub>2</sub>), 42.9 (quaternary C), 48.3 (CH), 55.3 (CH), 56.0 (CH), 71.4 (CH), 73.3 (CH), 125.5 (CH), 143.5 (quaternary C); *m/z* (ESI<sup>+</sup>) 399 [M + H – H<sub>2</sub>O]<sup>+</sup> (10%), 381 (70), 149 (20), 116 (100); HRMS calc for C<sub>28</sub>H<sub>47</sub>O [M + H – H<sub>2</sub>O]<sup>+</sup> 399.3627, found 399.3610. The following peaks were distinguishable for the minor campesterol component at a level <5%: 15.3 (CH<sub>3</sub>), 18.2 (CH<sub>3</sub>), 18.7 (CH<sub>3</sub>), 20.2 (CH<sub>3</sub>), 30.2 (CH<sub>2</sub>), 32.4 (CH), 35.8 (CH), 38.8 (CH), 55.4 (CH).

**5 $\beta$ ,6 $\beta$ -Epoxyergostan-3 $\beta$ -ol Acetate ( $\beta$ -Epoxide Acetate) (6).** Copper sulfate pentahydrate (3.164 g, 12.67 mM) and potassium permanganate (7.489 g, 47.40 mM) were ground together into a fine powder with a mortar and pestle to which water (1.4 mL) was added. The resulting paste was transferred to a flask containing DHB acetate (2) (0.700 g, 1.58 mM) in dichloromethane (20 mL). *t*-Butanol (0.84 mL) was added, and the reaction mixture was refluxed for 15 min before cooling to rt. The reaction mixture was then stirred for a further 16 h at rt. The reaction mixture was then filtered through a silica gel plug column eluting with dichloromethane. The product rich layer was then dried over magnesium sulfate and concentrated under reduced pressure to give a white solid. Purification by flash chromatography on silica gel [hexane:ethyl acetate (92.5:7.5)] yielded the title compound (6) as a white solid (0.360 g, 50%). <sup>1</sup>H NMR analysis ( $\delta_H$  3.07, d,  $\beta$ , 6-H and  $\delta_H$  2.89, d,  $\alpha$ , 6-H) showed this to be a mixture of the  $\beta$ - and  $\alpha$ -epoxides in a ratio of 5.3:1 which could not be separated by chromatography: mp 130–132 °C;  $[\alpha]_D^{20}$  –13.75° (*c* 1.000 in CHCl<sub>3</sub>);  $\nu_{\max}$ (KBr)/cm<sup>-1</sup> 2957, 2869, 1731, 1468, 1376;  $\delta_H$  (300 MHz, CDCl<sub>3</sub>) 0.61–2.15 [48H, m, containing 0.64 (3H, s, 18-CH<sub>3</sub>), 0.76–0.91

(12H, overlapping 4 × d, *J* 6.8, 21-CH<sub>3</sub>, 26-CH<sub>3</sub>, 27-CH<sub>3</sub> and 28-CH<sub>3</sub>), 1.00 (3H, s, 19-CH<sub>3</sub>), 2.03 (3H, s, COCH<sub>3</sub>), 3.07 (1H, bd, *J* 2.1, 6-H), 4.72–4.82 (1H, m, 3 $\alpha$ -H);  $\delta_C$  (75 MHz, CDCl<sub>3</sub>) 11.8 (CH<sub>3</sub>), 15.5 (CH<sub>3</sub>), 17.0 (CH<sub>3</sub>), 17.6 (CH<sub>3</sub>), 18.9 (CH<sub>3</sub>), 20.5 (CH<sub>3</sub>), 21.3 (CH<sub>3</sub>), 21.9 (CH<sub>2</sub>), 24.2 (CH<sub>2</sub>), 27.2 (CH<sub>2</sub>), 28.1 (CH<sub>2</sub>), 29.7 (CH), 30.5 (CH<sub>2</sub>), 31.5 (CH), 32.5 (CH<sub>2</sub>), 33.7 (CH<sub>2</sub>), 35.0 (quaternary C), 36.1 (CH), 36.7 (CH<sub>2</sub>), 38.0 (CH<sub>2</sub>), 39.1 (CH), 39.8 (CH<sub>2</sub>), 42.3 (quaternary C), 51.0 (CH), 56.0 (CH), 56.2 (CH), 62.5 (quaternary C), 63.6 (CH), 71.4 (CH), 170.5 (C=O); *m/z* (ESI<sup>+</sup>) 459 [M + H]<sup>+</sup> (8%), 441 (10), 399 (8), 149 (40), 116 (100); HRMS calc for C<sub>30</sub>H<sub>51</sub>O<sub>3</sub> [M + H]<sup>+</sup> 459.3838, found 459.3820. The following peaks were distinguishable for the minor campesterol component at a level <5%: 18.3 (CH<sub>3</sub>), 18.7 (CH<sub>3</sub>), 20.2 (CH<sub>3</sub>), 30.3 (CH<sub>2</sub>), 32.1 (CH<sub>2</sub>), 35.8 (CH), 38.8 (CH).

**5 $\beta$ ,6 $\beta$ -Epoxyergostan-3 $\beta$ -ol ( $\beta$ -Epoxide) (7).** A suspension of the  $\beta$ -epoxide acetate (6) (0.250 g, 0.55 mM) in methanol (50 mL) was stirred at rt for 5 min. Sodium carbonate (0.116 g, 1.09 mM) was added, and the mixture was stirred at rt for 16 h. The reaction mixture was then concentrated under reduced pressure to yield the crude product as a white solid. This product was purified by chromatography on silica gel using hexane–ethyl acetate (90:10) to give the epoxide (7) as a white solid (0.190 g, 83%). <sup>1</sup>H NMR analysis ( $\delta_H$  3.06, d,  $\beta$ , 6-H and  $\delta_H$  2.90, d,  $\alpha$ , 6-H) showed this to be a mixture of the  $\beta$ - and  $\alpha$ -epoxides in a ratio of 6.1:1 which could not be separated by chromatography: mp 132–134 °C;  $[\alpha]_D^{20}$  –7.10° (*c* 1.000 in CHCl<sub>3</sub>);  $\nu_{\max}$ (KBr)/cm<sup>-1</sup> 3436, 2957, 2869, 1466, 1377;  $\delta_H$  (300 MHz, CDCl<sub>3</sub>) 0.60–2.11 [46H, m, containing 0.61 (3H, s, 18-CH<sub>3</sub>), 0.76–0.90 (12H, overlapping 4 × d, *J* 6.8, 21-CH<sub>3</sub>, 26-CH<sub>3</sub>, 27-CH<sub>3</sub> and 28-CH<sub>3</sub>), 0.99 (3H, s, 19-CH<sub>3</sub>), 3.06 (1H, bd, *J* 2.1, 6-H), 3.63–3.73 (1H, m, 3 $\alpha$ -H);  $\delta_C$  (75 MHz, CDCl<sub>3</sub>) 11.8 (CH<sub>3</sub>), 15.5 (CH<sub>3</sub>), 17.1 (CH<sub>3</sub>), 17.6 (CH<sub>3</sub>), 18.9 (CH<sub>3</sub>), 20.5 (CH<sub>3</sub>), 22.0 (CH<sub>2</sub>), 24.2 (CH<sub>2</sub>), 28.1 (CH<sub>2</sub>), 29.8 (CH), 30.6 (CH<sub>2</sub>), 31.0 (CH<sub>2</sub>), 31.4 (CH), 32.6 (CH<sub>2</sub>), 33.7 (CH<sub>2</sub>), 34.9 (quaternary C), 36.1 (CH), 37.3 (CH<sub>2</sub>), 39.0 (CH), 39.8 (CH<sub>2</sub>), 42.2 (CH<sub>2</sub>), 42.3 (quaternary C), 51.3 (CH), 56.0 (CH), 56.2 (CH), 63.0 (quaternary C), 63.8 (CH), 69.3 (CH); *m/z* (ESI<sup>+</sup>) 417 [M + H]<sup>+</sup> (6%), 399 (46), 381 (20), 149 (20), 116 (100); HRMS calc for C<sub>28</sub>H<sub>49</sub>O<sub>2</sub> [M + H]<sup>+</sup> 417.3733, found 417.3730. The following peaks were distinguishable for the minor campesterol component at a level <5%: 18.3 (CH<sub>3</sub>), 18.7 (CH<sub>3</sub>), 20.2 (CH<sub>3</sub>), 35.8 (CH), 38.8 (CH).

**5 $\alpha$ ,6 $\alpha$ -Epoxyergostan-3 $\beta$ -ol ( $\alpha$ -Epoxide) (8).** A solution of *m*CPBA (70%, 0.518 g, 2.10 mM) in dichloromethane (30 mL) was added dropwise to a stirred solution of ice-cold DHB (1) (0.700 g, 1.75 mM) in dichloromethane (90 mL). The resulting mixture was stirred at rt for 2 h. The reaction mixture was then washed with 10% aqueous sodium hydrogen sulfite solution (2 × 50 mL), 5% aqueous sodium thiosulfate solution (2 × 50 mL), saturated aqueous sodium bicarbonate (2 × 100 mL), and aqueous sodium chloride (2 × 150 mL). The dichloromethane extracts were then dried over magnesium sulfate and concentrated under reduced pressure to yield the crude product as a white solid. This product was purified by chromatography on silica gel using hexane–ethyl acetate (80:20) to give the epoxide (8) as a white solid (0.648 g, 89%). <sup>1</sup>H NMR analysis ( $\delta_H$  2.90, d,  $\alpha$ , 6-H and  $\delta_H$  3.06, d,  $\beta$ , 6-H) showed this to be a mixture of the  $\alpha$ - and  $\beta$ -epoxides in a ratio of 6.6:1 which could not be separated by chromatography: mp 144–146 °C;  $[\alpha]_D^{20}$  –41.10° (*c* 1.000 in CHCl<sub>3</sub>);  $\nu_{\max}$ (KBr)/cm<sup>-1</sup> 3435, 2931, 2870 1631, 1467, 1377;  $\delta_H$  (300 MHz, CDCl<sub>3</sub>) 0.61 (3H, s, 18-CH<sub>3</sub>), 0.64–2.11 [43H, m, containing 0.76–0.90 (12H, overlapping 4 × d, *J* 6.8, 21-CH<sub>3</sub>, 26-CH<sub>3</sub>, 27-CH<sub>3</sub> and 28-CH<sub>3</sub>), 1.05 (3H, s, 19-CH<sub>3</sub>), 2.90 (1H, bd, *J* 4.2, 6-H), 3.84–3.93 (1H, m, 3 $\alpha$ -H);  $\delta_C$  (75 MHz, CDCl<sub>3</sub>) 11.8 (CH<sub>3</sub>), 15.4 (CH<sub>3</sub>), 15.9 (CH<sub>3</sub>), 17.5 (CH<sub>3</sub>), 18.8 (CH<sub>3</sub>), 20.5 (CH<sub>3</sub>), 20.6 (CH<sub>2</sub>), 24.0 (CH<sub>2</sub>), 28.0 (CH<sub>2</sub>), 28.7 (CH<sub>2</sub>), 29.8 (CH), 30.5 (CH<sub>2</sub>), 30.9 (CH<sub>2</sub>), 31.4 (CH), 32.4 (CH<sub>2</sub>), 33.6 (CH<sub>2</sub>), 34.8 (quaternary C), 36.1 (CH), 39.0 (CH), 39.3 (CH<sub>2</sub>), 39.7 (CH<sub>2</sub>), 42.3 (quaternary C), 42.5 (CH), 55.7 (CH), 56.8 (CH), 59.3 (CH), 65.9 (quaternary C), 68.4 (CH); *m/z* (ESI<sup>+</sup>) 417 [M + H]<sup>+</sup> (6%), 400 (10), 399 (30), 149 (40), 116 (100); HRMS calc for C<sub>28</sub>H<sub>49</sub>O<sub>2</sub> [M + H]<sup>+</sup> 417.3733, found 417.3743. The following peaks were distinguishable for the minor campesterol

component at a level <5%: 18.2 (CH<sub>3</sub>), 18.6 (CH<sub>3</sub>), 20.2 (CH<sub>3</sub>), 30.2 (CH<sub>2</sub>), 35.8 (CH), 38.7 (CH).

**Ergostane-3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -triol (Triol) (9).** To a solution of  $\alpha$ -epoxide (8) ( $\alpha$ : $\beta$ , 6.6:1, 0.470 g, 1.16 mM) in acetone (30 mL) and water (6 mL) was added 20 drops of concentrated sulfuric acid. The reaction mixture was stirred at rt for 2.5 h and was then concentrated. Ethyl acetate (30 mL) was added to the residue, which was then washed with water (2  $\times$  30 mL) and aqueous sodium chloride (30 mL) and dried over magnesium sulfate. The solvent was then evaporated under reduced pressure to yield the crude product as a white solid. This product was purified by chromatography on silica gel using hexane–ethyl acetate (30:70) to give the triol (9) as a white solid (0.363 g, 74%): mp 248–250 °C;  $[\alpha]_D^{20}$   $-4.9^\circ$  (*c* 1.000 in MeOH/H<sub>2</sub>O);  $\nu_{\max}$ (KBr)/cm<sup>-1</sup> 3433, 2956, 2870, 1466, 1384;  $\delta_H$  (300 MHz, DMSO-*d*<sub>6</sub>) 0.62 (3H, s, 18-CH<sub>3</sub>), 0.75–1.92 [42H, m containing 0.74–0.89 (12H, m, 21-CH<sub>3</sub>, 26-CH<sub>3</sub>, 27-CH<sub>3</sub> and 28-CH<sub>3</sub>), 1.02 (3H, s, 19-CH<sub>3</sub>)], 3.30 (1H, s, OH), 3.63 (1H, s, 6-H), 3.75–3.85 (1H, m, 3 $\alpha$ -H), 4.17 (1H, d, *J* 5.7, OH), 4.39 (1H, d, *J* 4.2, OH);  $\delta_C$  (150 MHz, CDCl<sub>3</sub>) 11.9 (CH<sub>3</sub>), 15.4 (CH<sub>3</sub>), 16.3 (CH<sub>3</sub>), 17.5 (CH<sub>3</sub>), 18.7 (CH<sub>3</sub>), 20.4 (CH<sub>3</sub>), 20.7 (CH<sub>2</sub>), 23.9 (CH<sub>2</sub>), 27.8 (CH<sub>2</sub>), 30.0 (CH), 30.1 (CH<sub>2</sub>), 30.8 (CH), 31.1 (CH<sub>2</sub>), 32.0 (CH<sub>2</sub>), 33.2 (CH<sub>2</sub>), 34.5 (CH<sub>2</sub>), 35.7 (CH), 37.8 (quaternary C), 38.4 (CH), 39.8 (CH<sub>2</sub>), 40.9 (CH<sub>2</sub>), 42.2 (quaternary C), 44.5 (CH), 55.6 (CH), 55.8 (CH), 65.8 (CH), 74.1 (CH), 74.3 (quaternary C); *m/z* (ESI<sup>+</sup>) 399 [M + H – 2H<sub>2</sub>O]<sup>+</sup> 10%, 381 (4), 149 (40), 116 (100); HRMS calc for C<sub>28</sub>H<sub>47</sub>O [M + H – 2H<sub>2</sub>O]<sup>+</sup> 399.3627, found 399.3646. The following peaks were distinguishable for the minor campesterol component at a level <5%: 15.3 (CH<sub>3</sub>), 18.1 (CH<sub>3</sub>), 18.5 (CH<sub>3</sub>), 20.0 (CH<sub>3</sub>), 29.8 (CH<sub>2</sub>), 31.8 (CH), 33.1 (CH<sub>2</sub>), 35.3 (CH), 38.1 (CH).

**Cell Treatment with Phytosterol Oxides.** U937 cells or HepG2 cells at a density of 2  $\times$  10<sup>5</sup> cells/mL were supplemented with reduced serum media (2.5% (v/v) FBS), before compound addition. DHB oxide derivatives were dissolved in ethanol and added to the cells at a final concentration of 30, 60, and 120  $\mu$ M. For comparison, cells were also incubated with 30  $\mu$ M of all available COPs, cholesterol-5 $\alpha$ ,6 $\alpha$ -epoxide ( $\alpha$ -epoxide), cholesterol-5 $\beta$ ,6 $\beta$ -epoxide ( $\beta$ -epoxide), 7-keto-cholesterol (7-keto), and 7- $\beta$ -OH-cholesterol (7- $\beta$ -OH). Each compound was dissolved in ethanol, and the final concentration in media did not exceed 0.4% (v/v). Equivalent quantities of ethanol were added to control cells, and samples were incubated for 24 h at 37 °C and 5% (v/v) CO<sub>2</sub>.

**Cell Viability Using MTT Assay.** U937 cells were seeded in the wells of a 96 well plate and were exposed to COPs and POPs for 24 h. According to the method previously outlined by Phelan et al.,<sup>25</sup> 10  $\mu$ L of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was added to each of the samples and they were incubated for a further 4 h prior to the addition of 100  $\mu$ L of solubilization solution (10% SDS in 0.01 M HCl). The plates were returned to the incubator overnight. The absorbance of each of the samples was determined at 570 nm with a reference wavelength of 690 nm using a Tecan Spectrafluor Plus plate reader. Data are expressed as percentage viability relative to the control ethanol treated sample.

**Cell Viability Using FDA/EtBr Assay or Neutral Red Uptake Assay.** The viability of U937 cells was assessed after 24 h treatment with POPs and COPs by the fluorescein diacetate/ethidium bromide assay. Briefly, cells were mixed 1:1 (v/v) with a solution of fluorescein diacetate and ethidium bromide and incubated at 37 °C for 5 min before being layered onto a microscope slide. Under these conditions, viable cells fluoresce green, while nonviable cells fluoresce red. Samples were examined at 200 $\times$  magnification on a Nikon fluorescence microscope using blue light (450–490 nm). Two hundred cells were scored for each condition, and cell viability was expressed as the percentage of viable (green) cells.

The viability of HepG2 cells was measured after 24 h using the Neutral Red uptake (NRU) assay. The NRU assay is a more suitable method for measuring cell viability in this adherent cell line. After 24 h incubation with the test compounds, medium was removed and the cells were washed with Krebs buffer solution. Neutral Red dye (50  $\mu$ g/mL) was added to each well of the 24 well plates and incubated for 2 h at 37 °C and 5% (v/v) CO<sub>2</sub> to allow uptake of the dye into lysosomes

of viable uninjured cells. The medium was removed, and cells were washed with Krebs buffer. The cells were subsequently treated with 1 mL of 1% glacial acetic acid/50% ethanol to burst open the cells and release the dye. After agitation for a few minutes, the absorbance of each sample was read on a plate reader at a wavelength of 540 nm.

**Morphological Analysis of Cell Nuclei.** Nuclear morphology of control, COPs-treated, and POPs-treated cells was assessed by fluorescence microscopy after staining with Hoechst 33342. The Hoechst method was conducted in U937 cells only. Approximately 4  $\times$  10<sup>5</sup> cells were centrifuged at 2000 rpm for 10 min to form a pellet. Hoechst 33342 stain (5  $\mu$ g/mL) was added, and the samples were incubated at 37 °C and 5% (v/v) CO<sub>2</sub> for 30 min. Stained samples were placed on a microscope slide and examined under UV light (330–380 nm) using a Nikon fluorescence microscope (400 $\times$  magnification). A total of 300 cells per sample were investigated, and the percentage of fragmented and condensed nuclei was calculated. Apoptotic cells were characterized by nuclear condensation of chromatin, nuclear fragmentation, and blebbing of the nucleus.

**DNA Fragmentation.** Detection of small DNA fragments was conducted as previously described.<sup>26</sup> DNA fragmentation was carried out in U937 cells only. Briefly, 2  $\times$  10<sup>6</sup> cells were harvested, the pellets were lysed, RNase A (0.25 mg/mL) was added, and the samples were incubated at 50 °C for 1 h. Proteinase K (5 mg/mL) was added, and the samples were incubated again at 50 °C for a further 1 h before being loaded into the wells of a 1.5% (w/v) agarose gel. A 100–1500 bp DNA standard (Promega; Medical Supply Co. Ltd., Dublin, Ireland) was used to assess the DNA fragmentation. Electrophoresis was carried out on agarose gel prepared in TBE buffer (0.45 M Tris, 0.45 M boric acid and 2 mM EDTA, pH 8) at 3 V/cm. DNA was visualized under UV light on a transilluminator (312 nm) after ethidium bromide staining and photographed using an image analysis system.

**Caspase-3 and Caspase-7 Activity.** The caspase-Glo 3/7 assay (Promega, Ireland) is a homogeneous, luminescent assay that measures total activity of both caspase-3 and caspase-7. These members of the cysteine aspartic acid-specific protease (caspase) family play key effector roles in apoptosis in mammalian cells. The assay provides a luminogenic caspase-3/7 substrate, which results in cell lysis, followed by caspase cleavage of the substrate and generation of a “glow-type” luminescent signal, produced by luciferase. Luminescence is proportional to the amount of caspase activity present. Briefly, U937 and HepG2 cells were seeded in the wells of a 96 well plate (HepG2 cells were allowed to adhere for 24 h) and exposed to COPs and POPs for 24 h. The Caspase-Glo reagent was added to the cells at a volume of 1:1, and the cells were incubated for a further 3 h. The luminescence of the samples was measured on a Tecan Spectrafluor Plus plate reader, and the data were expressed as fold increase relative to an untreated control sample.

**Statistical Analysis.** All data points are the mean and standard error values of at least three independent experiments. Data were analyzed by ANOVA followed by Dunnett's test. The software employed for statistical analysis was Prism (Hearne Scientific Software, Dublin, Ireland).

## RESULTS AND DISCUSSION

The benchmark compounds synthesized in this study are the 7-keto (4), 7- $\beta$ -OH (5),  $\beta$ -epoxide (7),  $\alpha$ -epoxide (8), and triol (9) of DHB (Figure 2) to form a comparative study of phytosterol oxides with previous studies.<sup>27,28</sup> The oxidation products were produced directly from a sample of DHB (1) of >95% purity synthesized previously.<sup>24</sup> The various oxidation products formed center around the B-ring at the 5-, 6-, and 7-positions in line with previous studies. DHB (1) was converted to its corresponding acetate (2) using standard acetylation conditions. Allylic oxidation employing chromium trioxide forms the acetate of 7-keto DHB (3). The acetate group is consequently cleaved using base-catalyzed hydrolysis conditions furnishing 7-keto DHB (4). The 7-keto steroid (4) was

stereoselectively reduced using sodium borohydride in the presence of cerium chloride heptahydrate. It has been reported that attack of a cerium ion on the carbonyl oxygen forms a cerium–steroid complex promoting attack of borohydride at the axial position allowing formation of the equatorial alcohol functional group.<sup>29</sup>

Stereoselective epoxidation was achieved using a biphasic system of potassium permanganate and copper sulfate in order to enhance oxidation on the more hindered  $\beta$ -face.<sup>30</sup> Removal of the acetate group affords  $\beta$ -epoxide (7). The epoxidation produced a mixture of epimers inseparable by chromatography where the  $\beta$ -epoxide predominates over the  $\alpha$ -epoxide in a ratio of 6.1:1 evident from <sup>1</sup>H NMR analysis ( $\delta_{\text{H}}$  3.06, d,  $\beta$  6-H and  $\delta_{\text{H}}$  2.90, d,  $\alpha$  6-H). Exposure of DHB (1) to *m*CPBA yields the  $\alpha$ -epoxide (8). The peracid approaches from the less hindered side of the double bond and produces a mixture of epimers where the  $\alpha$ -epoxide predominates over the  $\beta$ -epoxide in a ratio of 6.6:1 evident from <sup>1</sup>H NMR analysis ( $\delta_{\text{H}}$  2.90, d,  $\alpha$ , 6-H and  $\delta_{\text{H}}$  3.06, d,  $\beta$ , 6-H). To complete the panel of oxides, synthesis of triol (9) was accomplished. Acid-catalyzed ring-opening of  $\alpha$ -epoxide (8) provided a direct route to the desired triol (9). Once the panel of DHB oxides had been constructed, we proceeded to biological evaluation of these significant pure POPs.

COPs were added to U937 cells at a concentration of 30  $\mu\text{M}$ , and POPs were added at concentrations of 30  $\mu\text{M}$ , 60  $\mu\text{M}$ , and 120  $\mu\text{M}$ . The concentration of COPs in human plasma has been shown to be as high as 37  $\mu\text{M}$  following the ingestion of a test meal of spray-dried powdered eggs.<sup>31</sup> The concentrations of POPs employed in the present study were selected to allow a comparison with COPs which have typically been investigated at concentrations ranging from 10  $\mu\text{M}$  to 150  $\mu\text{M}$ .<sup>22,32</sup> In general, to induce a similar level of toxicity the concentrations of POPs required were approximately 3-fold higher than their COP counterparts.

When compared to control ethanol treated cells, the COPs investigated (30  $\mu\text{M}$  of  $\alpha$ -epoxide,  $\beta$ -epoxide, 7-keto, and 7- $\beta$ -OH) significantly reduced U937 viability as assessed by the MTT assay (Table 1). When each of the five oxidized derivatives of DHB were added to cells,  $\beta$ -epoxide at 60 and 120  $\mu\text{M}$  (7), 7-keto at 120  $\mu\text{M}$  (4), 7- $\beta$ -OH at 60 and 120  $\mu\text{M}$  (5), and all three concentrations of triol oxides (9) significantly reduced viability relative to control cells (Table 1). In an earlier study, a similar trend was observed for oxides of stigmaterol in U937 cells, whereby the  $\alpha$ -epoxide and  $\beta$ -epoxide were mildly cytotoxic, 7-keto showed a higher degree of toxicity, while triol (22*R*,23*R*) and 7- $\beta$ -OH were the most cytotoxic.<sup>17</sup> In relation to oxides of  $\beta$ -sitosterol, 7- $\alpha$ -OH sitosterol was the most cytotoxic to HepG2 cells as assessed by the MTT assay followed by 7-keto, while 7- $\beta$ -OH was mildly cytotoxic.<sup>16</sup> The authors suggest that toxicity of POPs is cell line specific as has previously been observed for COPs.<sup>23</sup> In U937 cells, three of the four COPs tested had a significant impact on cell viability ( $\beta$ -epoxide, 7-keto, and 7- $\beta$ -OH; Table 2) assessed using the FDA/EtBr assay. These results are in line with previous reported data.<sup>15,23,26</sup> Ryan et al. also assessed 3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -cholestane-triol (cholesterol triol) at 30  $\mu\text{M}$  and found U937 cell viability decreased to approximately 13% using the FDA-EtBr assay and viability decreased to approximately 11% in HepG2 cells when assessed by the NRU assay.<sup>15</sup> When the oxides of DHB were investigated, 7-keto at 120  $\mu\text{M}$  (4), 7- $\beta$ -OH at 60 and 120  $\mu\text{M}$  (5), and triol derivatives at all concentrations (dose-dependent) (9) caused significant cell

**Table 1. Cell Viability in U937 Cells Following Exposure for 24 h to 30, 60, or 120  $\mu\text{M}$  DHB Oxides (95% Purity)<sup>a</sup>**

compound	cell viability <sup>b</sup>	
	mean	SE
Cholesterol Oxides		
30 $\mu\text{M}$ $\alpha$ -epoxide	61.6**	2.5
30 $\mu\text{M}$ $\beta$ -epoxide	58.4**	9.9
30 $\mu\text{M}$ 7-keto	64.1**	9.3
30 $\mu\text{M}$ 7- $\beta$ -OH	49.4**	10.4
Phytosterol Oxides		
30 $\mu\text{M}$ $\alpha$ -epoxide	103.7	3.3
60 $\mu\text{M}$ $\alpha$ -epoxide	92.2	8.5
120 $\mu\text{M}$ $\alpha$ -epoxide	94.9	3.3
30 $\mu\text{M}$ $\beta$ -epoxide	81.2	4.4
60 $\mu\text{M}$ $\beta$ -epoxide	72.9*	3.7
120 $\mu\text{M}$ $\beta$ -epoxide	48.9**	2.0
30 $\mu\text{M}$ 7-keto	95.6	3.5
60 $\mu\text{M}$ 7-keto	76.2	8.1
120 $\mu\text{M}$ 7-keto	40.4**	7.3
30 $\mu\text{M}$ 7- $\beta$ -OH	117.1	14.6
60 $\mu\text{M}$ 7- $\beta$ -OH	39.3**	8.6
120 $\mu\text{M}$ 7- $\beta$ -OH	19.8**	6.0
30 $\mu\text{M}$ triol	44.1**	13.2
60 $\mu\text{M}$ triol	19.8**	5.2
120 $\mu\text{M}$ triol	8.2**	1.7

<sup>a</sup>The derivatives investigated were  $\alpha$ -epoxide (8),  $\beta$ -epoxide (7), 7-keto (4), 7- $\beta$ -OH (5), and triol (9). Cells were also treated with 30  $\mu\text{M}$  of the corresponding cholesterol oxide derivatives. All compounds were dissolved in ethanol, and equivalent quantities of ethanol (EtOH) were added to control cells. <sup>b</sup>Viability was assessed using the MTT assay, percentage value relative to ethanol treated cells. Values are means with their standard error of at least three independent experiments. Means values were significantly different from the control ethanol group (ANOVA followed by the Dunnett's test): \*  $P < 0.05$ , \*\*  $P < 0.01$ .

death ( $P < 0.01$ ) with the FDA-EtBr assay (Table 2). Similar findings were reported for the 7- $\beta$ -OH and triol derivatives of  $\beta$ -sitosterol and stigmaterol. However, the 7-keto derivative of  $\beta$ -sitosterol, but not stigmaterol, decreased U937 cell viability.<sup>15,17</sup> In the present study, the  $\alpha$ - and  $\beta$ -epoxide derivatives of DHB (8, 7) did not reduce cell viability in either cell line (Table 2). Previously, we reported the noncytotoxic effects in U937 cells of  $\alpha$ - and  $\beta$ -epoxide derivatives of stigmaterol and  $\beta$ -sitosterol.<sup>15,17</sup>

U937 cell viability values were lower with the MTT assay (Table 1) when compared to the FDA-EtBr assay (Table 2). The FDA-EtBr staining assay is a membrane integrity assay, and the MTT assay determines cell viability by measuring mitochondrial activity and can be a more sensitive measure of cytotoxicity.<sup>17</sup> Therefore, although a similar trend was observed for both viability assays, in that the relative toxicities of each of the compounds were similar, there was a more evident decrease in cell viability as measured by MTT. Cell viability following 24 h exposure to COPs and POPs was assessed in the HepG2 cell line using the NRU assay (Table 2). The NRU assay has previously been employed to assess viability in adherent cell lines.<sup>15</sup> Thus, we carried out this assay in HepG2 cell lines to allow us to compare our viability results after exposure to DHB oxides to previously published data investigating oxides generated from other phytosterol sources. Neutral Red is a weak, cationic, water-soluble dye that is only taken up by viable cells. In this study, the  $\beta$ -epoxide derivative of cholesterol

**Table 2. Percentage of Viable Cells Following Exposure for 24 h to 30, 60, or 120  $\mu\text{M}$  of DHB Oxides (95% Purity)<sup>a</sup>**

compound	cell viability <sup>b</sup>			
	U937 cells		HepG2 cells	
	mean	SE	mean	SE
control (EtOH)	95.3	0.3	100.0	0.0
Cholesterol Oxides				
30 $\mu\text{M}$ $\alpha$ -epoxide	89.1	2.4	95.8	3.6
30 $\mu\text{M}$ $\beta$ -epoxide	77.6**	2.8	73.3**	4.8
30 $\mu\text{M}$ 7-keto	77.7**	1.7	95.5	4.4
30 $\mu\text{M}$ 7- $\beta$ -OH	78.9**	2.9	85.4	10.9
Phytosterol Oxides				
30 $\mu\text{M}$ $\alpha$ -epoxide	90.1	1.1	100.1	2.0
60 $\mu\text{M}$ $\alpha$ -epoxide	86.5	3.7	98.2	2.6
120 $\mu\text{M}$ $\alpha$ -epoxide	89.4	2.6	103.6	3.1
30 $\mu\text{M}$ $\beta$ -epoxide	93.0	2.9	100.3	3.3
60 $\mu\text{M}$ $\beta$ -epoxide	89.5	4.0	99.9	3.9
120 $\mu\text{M}$ $\beta$ -epoxide	85.7	2.3	97.1	2.4
30 $\mu\text{M}$ 7-keto	89.5	3.7	93.7	2.8
60 $\mu\text{M}$ 7-keto	86.9	3.8	93.4	2.9
120 $\mu\text{M}$ 7-keto	44.3**	5.7	89.5	2.8
30 $\mu\text{M}$ 7- $\beta$ -OH	85.5	4.0	93.8	3.6
60 $\mu\text{M}$ 7- $\beta$ -OH	64.6**	7.7	88.8	3.7
120 $\mu\text{M}$ 7- $\beta$ -OH	34.0**	1.7	81.7**	3.4
30 $\mu\text{M}$ triol	72.7**	2.8	75.9**	3.6
60 $\mu\text{M}$ triol	44.2**	7.2	63.4**	2.8
120 $\mu\text{M}$ triol	7.0**	1.9	45.6**	2.1

<sup>a</sup>The derivatives investigated were  $\alpha$ -epoxide (8),  $\beta$ -epoxide (7), 7-keto (4), 7- $\beta$ -OH (5), and triol (9). Cells were also treated with 30  $\mu\text{M}$  of the corresponding cholesterol oxide derivatives. All compounds were dissolved in ethanol, and equivalent quantities of ethanol (EtOH) were added to control cells. Viability of the U937 cells was assessed by the fluorescein diacetate/ethidium bromide assay; viability of the HepG2 cells was assessed via the Neutral Red uptake assay. <sup>b</sup>Values are means with their standard error of at least three independent experiments. Means values were significantly different from the control ethanol group (ANOVA followed by the Dunnett's test): \*  $P < 0.05$ , \*\*  $P < 0.01$ .

significantly reduced cell viability to 73%. None of the other cholesterol derivatives significantly reduced HepG2 cell viability. In an earlier study, O'Callaghan et al. demonstrated that 7- $\beta$ -OH cholesterol is cytotoxic to HepG2 cells.<sup>26</sup> In the present study, the 7- $\beta$ -OH cholesterol did decrease cell viability (Table 2) but it did not reach statistical significance. Regarding the POPs, 7- $\beta$ -OH derivatives of DHB at 120  $\mu\text{M}$  (5) and all concentrations of triol (9) significantly reduced cell viability in HepG2 cells. Ryan et al. studied the viability of HepG2 and Caco-2 cells exposed to oxides of  $\beta$ -sitosterol (via the NRU assay).<sup>15</sup> They reported that the triol, 7-keto, and 7- $\beta$ -OH derivatives were cytotoxic to HepG2 cells and Caco-2 cells, while  $\alpha$ - and  $\beta$ -epoxide derivatives were noncytotoxic. Koschutnig et al. assessed the viability of HepG2 cells after exposure to single and mixed oxides of  $\beta$ -sitosterol using the trypan blue exclusion assay.<sup>16</sup> Cell viability showed a similar trend: 7- $\alpha$ -OH sitosterol had the greatest effect on viability followed by 7-keto sitosterol and 7- $\beta$ -OH sitosterol.

A number of studies have investigated the relationship between the structure of COPs and their biological effects. Generally, triol derivatives have been found to be the most cytotoxic to cells in culture, followed by 7- $\beta$ -OH,  $\beta$ -epoxide, 7-keto, and finally  $\alpha$ -epoxide.<sup>33</sup> It has been shown that 7- $\beta$ -OH

and  $\beta$ -epoxide were 10-fold more cytotoxic than their  $\alpha$ -isomers in human arterial endothelial cells.<sup>34</sup> The difference in the cytotoxicity of the various COPs and POPs may be caused by differences in their uptake and metabolism by cells. There are limited studies investigating the uptake of POPs in cells, however it has been found that  $\alpha$ -epoxide,  $\beta$ -epoxide, and triol oxides of cholesterol are all taken up at a similar rate by rabbit aortic endothelial cells.<sup>35</sup> COPs with similar structures have demonstrated different metabolisms:<sup>36</sup>  $\alpha$ -epoxide is a direct modulator of Liver-X-Receptors (LXR),<sup>37</sup> and bis-5,6 $\alpha$ -24-(S),25-epoxycholesterol is a selective LXR $\alpha$  modulator.<sup>38</sup> Additionally, the 3 $\beta$ -sulfated form of  $\alpha$ -epoxide cholesterol is an antagonist of LXR, whereas the  $\beta$ -epoxide equivalent is inactive.<sup>39</sup>

The morphology of U937 cells was examined under UV light following staining with Hoechst 33342 to help identify apoptotic cells. Nuclei, which were condensed or fragmented, were identified as apoptotic cells. Additionally, cells experiencing bulging of the membrane or "blebbing" were also defined as apoptotic. In control cells, apoptosis was approximately 11% (Table 3). This level appears high for control cells, but we included cells undergoing "blebbing" in our assessment of apoptosis. To confirm apoptosis in U937 cells, we also

**Table 3. Percentage of Condensed and Fragmented Nuclei in U937 Cells Following Exposure for 24 h to 30, 60, or 120  $\mu\text{M}$  DHB Oxides (95% Purity)<sup>a</sup>**

compound	% apoptosis <sup>b</sup>	
	mean	SE
control (EtOH)	11.3	1.0
Cholesterol Oxides		
30 $\mu\text{M}$ $\alpha$ -epoxide	21.3*	1.5
30 $\mu\text{M}$ $\beta$ -epoxide	28.1**	3.2
30 $\mu\text{M}$ 7-keto	32.5**	2.7
30 $\mu\text{M}$ 7- $\beta$ -OH	27.5**	2.6
Phytosterol Oxides		
30 $\mu\text{M}$ $\alpha$ -epoxide	18.6	1.7
60 $\mu\text{M}$ $\alpha$ -epoxide	18.8	1.6
120 $\mu\text{M}$ $\alpha$ -epoxide	21.7	2.6
30 $\mu\text{M}$ $\beta$ -epoxide	18.6	4.2
60 $\mu\text{M}$ $\beta$ -epoxide	20.4	1.3
120 $\mu\text{M}$ $\beta$ -epoxide	20.3	2.9
30 $\mu\text{M}$ 7-keto	20.4	4.2
60 $\mu\text{M}$ 7-keto	31.5**	3.1
120 $\mu\text{M}$ 7-keto	37.1**	5.4
30 $\mu\text{M}$ 7- $\beta$ -OH	21.4	2.0
60 $\mu\text{M}$ 7- $\beta$ -OH	23.6*	1.9
120 $\mu\text{M}$ 7- $\beta$ -OH	27.7**	4.4
30 $\mu\text{M}$ triol	18.3	3.7
60 $\mu\text{M}$ triol	19.3	1.2
120 $\mu\text{M}$ triol	18.9	5.1

<sup>a</sup>The derivatives investigated were  $\alpha$ -epoxide (8),  $\beta$ -epoxide (7), 7-keto (4), 7- $\beta$ -OH (5), and triol (9). Cells were also treated with 30  $\mu\text{M}$  of the corresponding cholesterol oxide derivatives. All compounds were dissolved in ethanol, and equivalent quantities of ethanol (EtOH) were added to control cells. The morphology of the nuclei was assessed using the Hoechst 33342 stain and the number of apoptotic nuclei expressed as a percentage of the total number. <sup>b</sup>Values are means with their standard error of at least three independent experiments. Means values were significantly different from the control ethanol group (ANOVA followed by the Dunnett's test): \*  $P < 0.05$ , \*\*  $P < 0.01$ .

measured caspase activities and DNA fragmentation (see below). All COPs significantly increased apoptosis in U937 cells relative to control as assessed by Hoechst staining (Table 3). For the POPs, 7-keto (4) and 7- $\beta$ -OH (5) at 60  $\mu$ M and 120  $\mu$ M appeared to induce apoptosis in U937 cells (Table 3). The triol derivatives (9), while very cytotoxic (Tables 1 and 2), were nonapoptotic and therefore we assume were killing the cells by necrosis. Necrosis is unprogrammed cell death, where dying cells are not engulfed by phagocytes but instead fragment into many pieces of debris, leading to disorganized and detrimental breakdown of the cell. Necrosis was not quantified in the present study.

It is evident that, in U937 cells exposed to 60  $\mu$ M 7-keto oxide, the degree of apoptosis appears to be greater than the degree of cell death: 13% cell death (FDA/EtBr assay) with 31.5% apoptosis. FDA/EtBr is a marker of cell integrity; therefore it is plausible that cell integrity was not dramatically affected but cells were still able to enter the early stages of apoptosis. This effect was not evident at 120  $\mu$ M. It is apparent that, in general, the  $\alpha$ -epoxide (8) and  $\beta$ -epoxide (7) of DHB, as well as the corresponding derivatives of  $\beta$ -sitosterol and stigmasterol, are nonapoptotic.<sup>15,17</sup> 7-Keto-stigmasterol did not induce apoptosis in U937 cells;<sup>17</sup> however apoptosis did appear to be induced in the present study after exposure to high concentrations: 60 and 120  $\mu$ M of the 7-keto oxide (4). The 7- $\beta$ -OH derivatives of  $\beta$ -sitosterol and stigmasterol<sup>15,17</sup> as well as the oxide of DHB (5) appear to be apoptotic as measured by Hoechst staining.

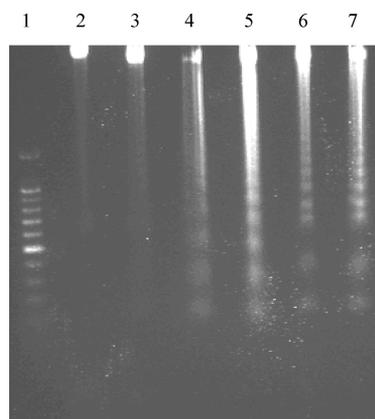
Caspase activity was significantly increased after U937 cells were exposed to 30  $\mu$ M of the COPs,  $\beta$ -epoxide and 7-keto (Table 4). In relation to the DHB oxides, caspase activity in U937 cells was significantly increased following exposure to 60 and 120  $\mu$ M 7- $\beta$ -OH (5). Similarly, the oxides of stigmasterol including 7- $\beta$ -OH increased caspase activity in U937 cells.<sup>17</sup> Apoptotic potential of POPs in the U937 cell line was verified by DNA laddering (Figure 3). It is evident that the 7-keto oxide did show an increase in apoptosis with the Hoechst method but did not increase caspase activity in U937 cells. Morphological changes may be evident indicating apoptosis is occurring, but caspase activity may be unaffected as the compound may be inducing cell death by caspase-independent mechanisms. Prunet et al. demonstrated that 7-keto and 7- $\beta$ -OH cholesterol can induce cell death by both caspase-dependent and -independent pathways.<sup>40</sup> Therefore, their equivalent DHB oxides may have the potential to induce cell death through caspase-independent pathways.

Unlike macrophage cell lines such as U937 cells, previous studies have demonstrated that COPs are cytotoxic but do not induce apoptosis in HepG2 cells.<sup>15,23</sup> Therefore, only caspase activity was assessed in the HepG2 cell line (Table 4) following exposure to COPs and POPs. COPs had no impact on caspase activity in this cell line. Of the DHB oxides, only triol (60 and 120  $\mu$ M) significantly increased caspase activity in HepG2 cells. Biasi et al. investigated the apoptotic potential of a mix of COPs in Caco-2 cells.<sup>9</sup> It was apparent that COPs were proapoptotic in differentiated cells but not in the undifferentiated state. Exposure of differentiated Caco-2 cells to COPs resulted in significantly stronger caspase activity than that of unoxidized cholesterol. Koschutnig et al. assessed the apoptotic potential of single and mixed oxides of  $\beta$ -sitosterol in the HepG2 cell line, and only 7-keto sitosterol appeared to have apoptotic properties.<sup>16</sup> In contrast, oxidation products of  $\beta$ -sitosterol

**Table 4. Caspase Activity Following Exposure for 24 h to 30, 60, or 120  $\mu$ M DHB Oxides (95% Purity)<sup>a</sup>**

compound	caspase act. (fold increase rel to control ethanol) <sup>b</sup>			
	U937 cells		HepG2 cells	
	mean	SE	mean	SE
Cholesterol Oxides				
30 $\mu$ M $\alpha$ -epoxide	1.2	0.1	1.1	0.1
30 $\mu$ M $\beta$ -epoxide	2.0*	0.4	1.3	0.2
30 $\mu$ M 7-keto	1.9*	0.2	1.0	0.1
30 $\mu$ M 7- $\beta$ -OH	1.4	0.2	1.2	0.2
Phytosterol Oxides				
30 $\mu$ M $\alpha$ -epoxide	1.0	0.1	1.1	0.1
60 $\mu$ M $\alpha$ -epoxide	1.1	0.1	1.1	0.1
120 $\mu$ M $\alpha$ -epoxide	1.5	0.3	1.0	0.1
30 $\mu$ M $\beta$ -epoxide	0.9	0.1	0.9	0.0
60 $\mu$ M $\beta$ -epoxide	1.3	0.2	1.0	0.1
120 $\mu$ M $\beta$ -epoxide	1.8	0.5	1.1	0.1
30 $\mu$ M 7-keto	1.0	0.0	0.9	0.1
60 $\mu$ M 7-keto	1.6	0.3	0.9	0.0
120 $\mu$ M 7-keto	1.5	0.2	1.0	0.1
30 $\mu$ M 7- $\beta$ -OH	1.3	0.1	0.9	0.0
60 $\mu$ M 7- $\beta$ -OH	2.3*	0.3	1.2	0.1
120 $\mu$ M 7- $\beta$ -OH	3.0**	0.5	1.2	0.2
30 $\mu$ M triol	1.1	0.2	1.5	0.1
60 $\mu$ M triol	1.3	0.2	2.5**	0.2
120 $\mu$ M triol	1.6	0.5	4.0**	0.4

<sup>a</sup>The derivatives investigated were  $\alpha$ -epoxide (8),  $\beta$ -epoxide (7), 7-keto (4), 7- $\beta$ -OH (5), and triol (9). Cells were also treated with 30  $\mu$ M of the corresponding cholesterol oxide derivatives. All compounds were dissolved in ethanol, and equivalent quantities of ethanol (EtOH) were added to control cells. Caspase activity was expressed as fold increase relative to control ethanol cells. <sup>b</sup>Caspase activity is total caspase activity (caspase 3 and caspase 7) expressed as a percentage of ethanol control. Values are means with their standard error of at least three independent experiments. Mean values were significantly different from the control ethanol group (ANOVA followed by the Dunnett's test): \*  $P < 0.05$ , \*\*  $P < 0.01$ .



**Figure 3.** DNA fragmentation in U937 cells exposed to 120  $\mu$ M DHB oxides for 24 h. Lane 1: molecular weight marker (200 bp). Lane 2: control, ethanol treated cells. Lane 3:  $\alpha$ -epoxide (8). Lane 4:  $\beta$ -epoxide (7). Lane 5: 7-keto (4). Lane 6: 7- $\beta$ -OH (5). Lane 7: triol (9).

investigated by Ryan et al. were nonapoptotic in HepG2 and Caco-2 cell lines.<sup>15</sup>

This report discloses for the first time the synthesis and cytotoxicity of pure oxides of the significant phytosterol DHB and as such gives invaluable information on the toxicity profile

of an important component in the diet. Certain oxidized derivatives of DHB are cytotoxic in U937 and HepG2 cells. Overall, they appear to be more cytotoxic to U937 cells. Toxicity differed with each individual oxide investigated: 7-keto, 7- $\beta$ -OH, and triol derivatives show significant cytotoxic effects, and of these derivatives, 7- $\beta$ -OH appears to have the most apoptotic potential. In contrast,  $\alpha$ - and  $\beta$ -epoxide derivatives are relatively noncytotoxic and nonapoptotic. All the oxidized derivatives investigated in this study have a similar cytotoxic potential in U937 cells to previously reported POPs equivalent ( $\beta$ -sitosterol and stigmasterol oxide). Results generated in this study add to the data on the toxicity profile of individual POPs and help determine the hierarchy of toxicity of POPs.

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### Notes

The authors declare no competing financial interest.

## REFERENCES

- (1) Gupta, A. K.; Savopoulos, C. G.; Ahuja, J.; Hatzitolios, A. I. Role of phytosterols in lipid-lowering: current perspectives. *Q. J. Med.* **2011**, *104*, 301–308.
- (2) Lea, L. J.; Hepburn, P. A.; Wolfreys, A. M.; Baldrick, P. Safety evaluation of phytosterol esters. Part 8. Lack of genotoxicity and subchronic toxicity with phytosterol oxides. *Food Chem. Toxicol.* **2004**, *42*, 771–783.
- (3) Grandgirard, A.; Martine, L.; Joffre, C.; Juaneda, P.; Berdeaux, O. Gas chromatographic separation and mass spectrometric identification of mixtures of oxyphytosterol and oxysterol derivatives – application to a phytosterol-enriched food. *J. Chromatogr., A* **2004**, *1040*, 239–250.
- (4) Johnsson, L.; Dutta, P. C. Determination of phytosterol oxides in some food products by using an optimized transesterification method. *Food Chem.* **2006**, *97*, 606–613.
- (5) González-Larena, M.; García-Llatas, G.; Vidal, M.; Sánchez-Siles, L.; Barberá, R.; Lagarda, M. Stability of plants sterols in ingredients used in functional foods. *J. Agric. Food Chem.* **2011**, *59*, 3624–3631.
- (6) García-Llatas, G.; Rodríguez-Estrada, M. T. Current and new insights on phytosterol oxides in plant sterol-enriched food. *Chem. Phys. Lipids* **2011**, *164*, 607–624.
- (7) Tonello, A.; Poli, G. Serum phytosterols not only from dietary intake. *Br. J. Nutr.* **2006**, *96*, 791–792.
- (8) Wielkoszyński, T.; Gawron, K.; Strzelczyk, J.; Bodzek, P.; Zalewska-Ziob, M.; Trapp, G.; Srebniak, M.; Wiczowski, A. Cellular toxicity of oxysterols. *Bioessays* **2006**, *28*, 387–398.
- (9) Biasi, F.; Mascia, C.; Astegiano, M.; Chiarotto, E.; Nano, M.; Vizio, B.; Leonarduzzi, G.; Poli, G. Pro-oxidant and proapoptotic effects of cholesterol oxidation products on human colonic epithelial cells: a potential mechanism of inflammatory bowel disease progression. *Free Radical Biol. Med.* **2009**, *47*, 1731–1741.
- (10) Lordan, S.; Mackrill, J. J.; O'Brien, N. M. Oxysterols and mechanisms of apoptotic signaling: implications in the pathology of degenerative diseases. *J. Nutr. Biochem.* **2009**, *20*, 321–336.
- (11) Hovenkamp, E.; Demonty, I.; Plat, J.; Lütjohann, D.; Mensink, R. P.; Trautwein, E. A. Biological effects of oxidized phytosterols: a review of the current knowledge (review). *Prog. Lipid Res.* **2008**, *47*, 37–49.
- (12) Maguire, L.; Konoplyannikov, M.; Ford, A.; Maguire, A. R.; O'Brien, N. M. Comparison of the cytotoxic effects of beta-sitosterol oxides and a cholesterol oxide, 7-beta-hydroxycholesterol, in cultured mammalian cells. *Br. J. Nutr.* **2003**, *90*, 767–775.
- (13) Adcox, C.; Boyd, L.; Oehrl, L.; Allen, J.; Fenner, G. Comparative effects of phytosterol oxides and cholesterol oxides in cultured macrophage-derived cell lines. *J. Agric. Food Chem.* **2001**, *49*, 2090–2095.
- (14) Roussi, S.; Winter, A.; Gosse, F.; Werner, D.; Zhang, X.; Marchioni, E.; Geoffroy, P.; Miesch, M.; Raul, F. Different apoptotic mechanisms are involved in the antiproliferative effects of 7- $\beta$ -hydroxysitosterol and 7- $\beta$ -hydroxycholesterol in human colon cancer cells. *Cell Death Differ.* **2005**, *12*, 128–135.
- (15) Ryan, E.; Chopra, J.; McCarthy, F.; Maguire, A. R.; O'Brien, N. M. Qualitative and quantitative comparison of the cytotoxic and apoptotic potential of phytosterol oxidation products with their corresponding cholesterol oxidation products. *Br. J. Nutr.* **2005**, *94*, 443–451.
- (16) Koschutnig, K.; Heikkinen, S.; Kemmo, S.; Lampi, A. M.; Piironen, V.; Wagner, K. H. Cytotoxic and apoptotic effects of single and mixed oxides of beta-sitosterol on HepG2 cells. *Toxicol. In Vitro* **2009**, *23*, 755–762.
- (17) O'Callaghan, Y. C.; Foley, D. A.; O'Connell, N. M.; McCarthy, F. O.; Maguire, A. R.; O'Brien, N. M. Cytotoxic and apoptotic effects of the oxidised derivatives of stigmasterol in the U937 human monocytic cell line. *J. Agric. Food Chem.* **2010**, *58*, 10793–10798.
- (18) Misharin, A. Y.; Mehtiev, A. R.; Morozevich, G. E.; Tkachev, Y. V.; Timofeev, V. P. Synthesis and cytotoxicity evaluation of 22,23-oxygenated stigmastane derivatives. *Bioorg. Med. Chem.* **2008**, *16*, 1460–1473.
- (19) Patterson, G. W.; Krauss, R. W. Sterols of *Chlorella*. I. The naturally occurring sterols of *Chlorella vulgaris*, *C. ellipsoidea*, and *C. saccharophila*. *Plant Cell Physiol.* **1965**, *6*, 211–220.
- (20) Thompson, M. J.; Cohen, C. F.; Lancaster, S. M. Brassicasterol and 22, 23-dihydrobrassicasterol from i-ergosterol via -ergosterol. *Steroids* **1965**, *5*, 745–752.
- (21) Phillips, K. M.; Ruggio, D. M.; Toivo, J. I.; Swank, M. A.; Simpkins, A. H. Free and esterified sterol composition of edible oils and fats. *J. Food Compos. Anal.* **2002**, *15*, 123–142.
- (22) Lizard, G.; Gueldry, S.; Sordet, O.; Monier, S.; Athias, A.; Miguet, C.; Bessedé, G.; Lemaire, S.; Solary, E.; Gambert, P. Glutathione is implied in the control of 7-ketocholesterol-induced apoptosis, which is associated with radical oxygen species production. *FASEB J.* **1998**, *15*, 1651–1663.
- (23) O'Callaghan, Y. C.; Woods, J. A.; O'Brien, N. M. Characteristics of 7 $\beta$ -hydroxycholesterol-induced cell death in a human monocytic blood cell line, U937 and a human hepatoma cell line, HepG2. *Toxicol. In Vitro* **2002**, *16*, 245–251.
- (24) O'Connell, N.; O'Callaghan, Y. C.; O'Brien, N. M.; Maguire, A. R.; McCarthy, F. O. Synthetic routes to campesterol and dihydrobrassicasterol: a first reported synthesis of dihydrobrassicasterol. *Tetrahedron* **2012**, *68*, 4995–5004.
- (25) Phelan, M.; Aherne, S. A.; Wong, A.; O'Brien, N. M. Bioactive properties of wood knot extracts on cultured human cells. *J. Med. Food* **2009**, *12*, 1245–1251.
- (26) O'Callaghan, Y. C.; Woods, J. A.; O'Brien, N. M. Oxysterol-induced cell death in U937 and HepG2 cells at reduced and normal serum concentrations. *Eur. J. Nutr.* **1999**, *38*, 255–262.
- (27) McCarthy, F. O.; Chopra, J.; Ford, A.; Hogan, S. A.; Kerry, J. P.; O'Brien, N. M.; Ryan, E.; Maguire, A. R. Synthesis, isolation and characterisation of  $\beta$ -sitosterol and  $\beta$ -sitosterol oxide derivatives. *Org. Biomol. Chem.* **2005**, *3*, 3059–3065.
- (28) Foley, D. A.; O'Callaghan, Y.; O'Brien, N. M.; McCarthy, F. O.; Maguire, A. R. Synthesis and characterisation of stigmasterol oxidation products. *J. Agric. Food Chem.* **2010**, *58*, 1165–1173.
- (29) Štátná, E.; Černý, I.; Pouzar, V.; Chodounská, H. Stereoselectivity of sodium borohydride reduction of saturated steroidal ketones utilizing conditions of Luche reduction. *Steroids* **2010**, *75*, 721–725.
- (30) Salvador, J. A. R.; Sáe Melo, M. L.; Campos Neves, A. S. Oxidations with potassium permanganate—metal sulphates and nitrates.  $\beta$ -Selective epoxidation of  $\Delta^5$ -unsaturated steroids. *Tetrahedron Lett.* **1996**, *37*, 687–690.

(31) Emanuel, H. A.; Hassel, C. A.; Addis, P. B.; Bergman, S. D.; Zavoral, J. H. Plasma cholesterol oxidation products (or sterols) in human subjects fed a meal rich in oxysterols. *J. Food Sci.* **1991**, *56*, 843–847.

(32) Lizard, G.; Monier, S.; Cordelet, C.; Gesquière, L.; Deckert, V.; Gueldry, S.; Lagrost, L.; Gambert, P. Characterisation and comparison of the mode of cell death, apoptosis versus necrosis, induced by 7 $\beta$ -hydroxycholesterol and 7-ketocholesterol in the cells of the vascular wall. *Arterioscler., Thromb., Vasc. Biol.* **1999**, *19*, 1190–1200.

(33) Carvalho, J. F.; Silva, M. M.; Moreira, J. N.; Simões, S.; Sá e Melo, M. L. Sterols as anticancer agents: synthesis of ring-B oxygenated steroids, cytotoxic profile and comprehensive SAR analysis. *J. Med. Chem.* **2010**, *53*, 7632–7638.

(34) Rimmer, A.; Al Makdessi, S.; Sweidan, H.; Wischhusen, J.; Rabenstein, B.; Shatat, K.; Mayer, P.; Spyridopoulos, I. Relevance and mechanism of oxysterol stereospecificity in coronary artery disease. *Free Radical Biol. Med.* **2005**, *38*, 535–544.

(35) Sevanian, A.; Berliner, J.; Peterson, H. Uptake, metabolism, and cytotoxicity of isomeric cholesterol-5,6-epoxides in rabbit aortic endothelial cells. *J. Lipid Res.* **1991**, *32*, 147–155.

(36) Paillasse, M. R.; Saffon, N.; Gornitzka, H.; Silvente-Poirot, S.; Poirot, M.; de Medina, P. Surprising “un” reactivity of cholesterol-5,6-epoxides towards nucleophiles. *J. Lipid Res.* **2012**, *53*, 718–725.

(37) Berrodin, T. J.; Shen, Q.; Quinet, E. M.; Yudt, M. R.; Freedman, L. P.; Nagpal, S. Identification of 5 $\alpha$ , 6 $\alpha$ -epoxycholesterol as a novel modulator of liver X receptor activity. *Mol. Pharmacol.* **2010**, *78*, 1046–1058.

(38) Janowski, B. A.; Grogan, M. J.; Jones, S. A.; Wisely, G. B.; Kliewer, S. A.; Corey, E. J.; Mangelsdorf, D. J. Structural requirements of ligands for the oxysterol liver X receptors LXR $\alpha$  and LXR $\beta$ . *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 266–271.

(39) Song, C.; Hiipakka, R. A.; Liao, S. Auto-oxidized cholesterol sulfates are antagonistic ligands of liver X receptors: implications for the development and treatment of atherosclerosis. *Steroids* **2001**, *66*, 473–479.

(40) Prunet, C.; Lemaire-Ewing, S.; Ménétrier, F.; Néel, D.; Lizard, G. Activation of caspase-3-dependent and -independent pathways during 7-ketocholesterol- and 7 $\beta$ -hydroxycholesterol-induced cell death: a morphological and biochemical study. *J. Biochem. Mol. Toxicol.* **2005**, *19*, 311–326.