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Cytotoxic and Apoptotic Effects of the Oxidized Derivatives of Stigmasterol in the U937 Human Monocytic Cell Line

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Dietary exposure to phytosterols has increased in recent years due to the incorporation of these compounds into cholesterol-lowering products. Previous studies have investigated the cytotoxic effects of the oxidized derivatives of β-sitosterol and determined that phytosterol oxidation products (POP) have a similar but less potent toxicity compared to their cholesterol equivalents. In the present study, the cytotoxicity of the oxidized derivatives of stigmasterol were investigated in the U937 cell line. The stigmasta-5,22-diene-3β,7β-diol (7β-OH), 5,6-epoxystigmasta-22,23-diol (epoxydiol), 5,6,22,23-diepoxystigmastane (diepoxide), and (22*R*,23*R*)-stigmast-5-ene-3β,22,23-triol (22*R*,23*R*-triol) derivatives were identified as the most cytotoxic, and the mode of cell death was identified as apoptosis in cells incubated with 7β-OH, epoxydiol, and diepoxide stigmasterol. The antioxidants α-tocopherol, γ-tocopherol, and β-carotene did not protect against apoptosis induced by 7β-OH and diepoxide stigmasterol; however, α-tocopherol was found to protect against epoxydiol-induced apoptosis. The cellular antioxidant, glutathione, was depleted and the apoptotic protein, Bcl-2, was down-regulated by the stigmasterol oxides identified as apoptotic.

KEYWORDS: U937; stigmasterol oxidation products; cytotoxicity; apoptosis

INTRODUCTION

The plant-derived phytochemicals, phytosterols, are structurally similar to cholesterol with a double bond at the C_{5-6} position, which makes them susceptible to oxidation (*1*). The potential health risk posed by dietary exposure to cholesterol oxides (COP) has been a subject of investigation for a number of years (*2*, *3*). With the increasing incorporation of phytosterols into cholesterol-lowering products and the consequent increase in dietary consumption, it is important that the potential toxicity of phytosterol oxides (POP) be examined. A number of studies have investigated the toxic effects of POP, generated through thermo-oxidation, photo-oxidation, or chemical-induced oxidation processes, in cell model systems. The POP demonstrated cytotoxic effects, but the significance of the data is limited as the test compounds represent POP mixes and not pure compounds (*4*−*6*). To date, the unavailability of standards for individual phytosterol oxides has hampered both their quantification in foods and the assessment of their relative toxicity and potential atherogenic effects. A previous study undertaken by our research group examined the cytotoxicity of the individual oxides of β-sitosterol, synthesized as outlined in McCarthy et al. (*7*), and found that they induced cell death and apoptosis in a number of cell lines in a manner similar to but less marked than that of their COP equivalents (*8*).

The present study is an addendum to the work previously published by Foley et al. (*9*), in which a series of stigmasterol oxides were synthesized and characterized. Stigmasterol is among the three most prevalent of the phytosterols, the others being β-sitosterol and campesterol. Stigmasterol differs structurally from cholesterol, β-sitosterol, and campesterol in that it contains an additional double bond, positioned at C_{22-23} (*9*). The presence of the additional double bonds allows for the formation of oxides such as diepoxide and epoxydiol, compounds that do not have cholesterol or β-sitosterol equivalents.

Stigmasterol is generally present in foods at concentrations lower than those of β-sitosterol and campesterol; nevertheless, stigmasterol oxides have been detected in phytosterol-enriched spreads and oils such as peanut, sunflower, and corn oils (*10*, *11*). There is little information available on the absorption of phytosterol oxides in vivo; although oxides of β-sitosterol and campesterol have been detected in the plasma of healthy individuals, it remains to be elucidated whether the POP were absorbed from the diet or were oxidized in vivo (*12*).

The objective of the present study was to screen a range of oxidized derivatives of stigmasterol, 5α,6α-epoxystigmast-22-en-3β-ol (α-epoxide, **1**), 5β,6β-epoxystigmast-22-en-3β-ol (β-epoxide, **2**), stigmasta-5,22-dien-7-on-3β-ol (7-keto, **3**), stigmasta-5,22-diene-3β,7β-diol (7β-OH, **4**), 5,6-epoxystigmasta-22*S*,23*S*-diol (epoxydiol, **5**), 5,6,22,23-diepoxystigmastane (diepoxide, **6**), (22*R*,23*R*)-stigmast-5-ene-3β,22,23-triol (22*R*,23*R*-triol, **7**), and 5α-stigmast-22-ene-3β,5α,6β-triol (3,5,6-triol, **8**) for their cytotoxic and apoptotic effects in the U937 monocytic blood cell line (**Figure 1**). The individual oxides were synthesized and characterized according to the method outlined by Foley et al. (*9*). Antioxidants such as

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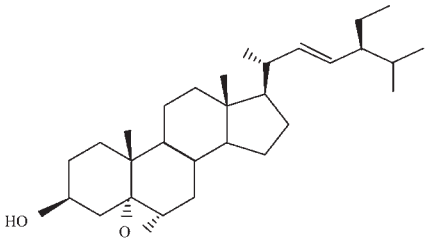
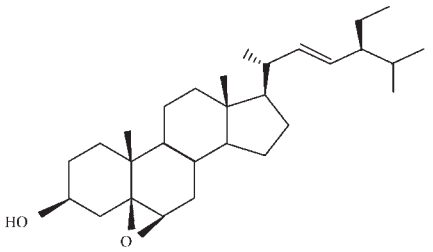


Compound 1: 5α,6α-epoxystigmast-22-en-3β-ol (5)



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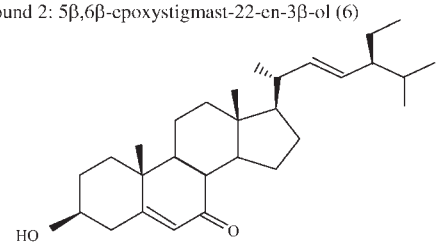
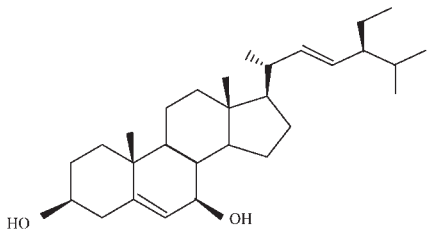
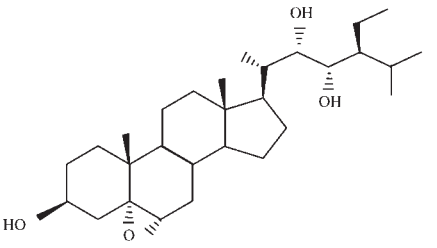


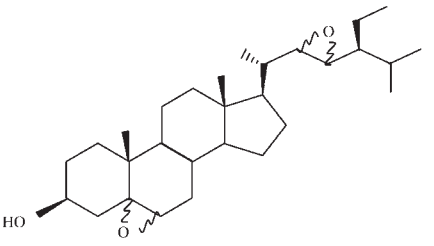
Compound 3: stigmasta-5,22-dien-7-on-3β-ol (7)



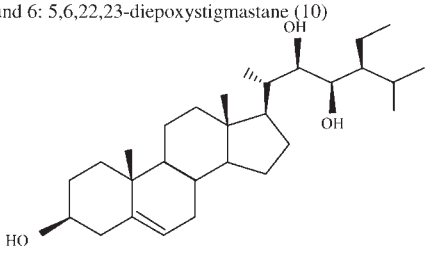
Compound 4: stigmasta-5,22-diene-3β,7β-diol (8)



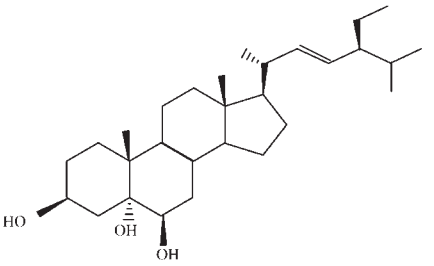
Compound 5: 5,6-epoxystigmasta-22,23-diol (21)



Compound 6: 5,6,22,23-diepoxystigmastane (10)



Compound 7: (22R,23R)-stigmast-5-ene-3β,22,23-triol (13)



Compound 8: 5α-stigmast-22-ene-3β,5α,6β-triol (9)

Figure 1. Structures of the stigmasterol oxides investigated in the present study. Numbers in parentheses denote numbers assigned to the compounds in Foley et al. (*9*).

α-tocopherol and γ-tocopherol have previously been shown to have protective effects against the cytotoxic effects of COP and POP (*8*). Therefore, a further objective of the study assessed the ability of α-tocopherol, γ-tocopherol, and β-carotene to protect against POP-induced apoptosis. Apoptotic indices including glutathione depletion, caspase-3 activity, and Bcl-2 levels were also assessed.

MATERIALS AND METHODS

Materials. All chemicals and reagents were purchased from Sigma-Aldrich Chemical Co. (Dublin, Ireland) unless otherwise stated. The U937 cells were obtained from the European Collection of Animal Cell Cultures (Salisbury, U.K.). Information relating to the stigmasterol oxides may be obtained in Foley et al. (*9*).

Maintenance of Cells in Culture. Human monocytic U937 cells were grown in suspension in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS). Cells were cultured in the absence of antibiotics. The cells were maintained at 37 °C/5% CO_2 in a humidified incubator and were subcultured every 2−3 days. Exponentially growing cells were used throughout. For treatment, cells were adjusted to a density of 1×10^5 cells/mL in media containing reduced (2.5%) FBS. The stigmasterol oxides were dissolved in ethanol and were added to the cells to a final concentration of 30, 60, or 120 μM. An equivalent volume of ethanol was added to the control cells. Antioxidants α-tocopherol and γ-tocopherol were dissolved in ethanol and added at a final concentration of 10 μM; β-carotene was dissolved in hexane and added to the cells at a concentration of 2 μM.

Cell Viability. The viability of the cells was assessed, following 24 h of incubation with the stigmasterol oxides, using a fluorochrome-mediated viability assay previously described by Ryan et al. (*13*). Briefly, cells were mixed 1:1 (v/v) with a solution of fluorescein diacetate (FDA) and ethidium bromide (EtBr) and then incubated at 37 °C for 5 min before being layered onto a microscope slide. Under these conditions live cells fluoresce green, whereas dead cells fluoresce red. Samples were examined at 200× magnification on a Nikon fluorescence microscope using a blue (450−490 nm) filter. Cells (200) were scored for each slide, and the cell viability was expressed as the percentage of viable (green) cells.

Cell Proliferation. Cells were seeded in the wells of a 96-well plate and were exposed to POP for 24 h. According to the method previously outlined in Phelan et al. (*14*), 10 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each of the samples, which were incubated for a further 4 h, prior to the addition of 100 μL 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 using a Tecan Spectrafluor Plus platereader. Data are expressed as a percentage of the untreated control sample.

Morphological Analysis of Cell Nuclei. The nuclear morphology of stigmasterol oxide treated cells was assessed by fluorescence microscopy following staining with Hoechst 33342 as previously described by Ryan et al. (*13*). Approximately 4×10^5 treated cells were harvested by centrifugation (200*g*, 10 min). Hoechst 33342 stain (200 μL, 5 μg/mL PBS) was added, and the samples were incubated at 37 °C/5% CO_2 for 1 h. Stained samples were placed on a microscope slide and examined under UV light (330−380 nm) using a Nikon fluorescence microscope (400× magnification). A total of 300 cells were analyzed for each sample, and the percentage of condensed/fragmented (apoptotic) nuclei was calculated.

DNA Fragmentation Assay. Detection of small DNA fragments was carried out as previously described by Ryan et al. (*13*). Briefly, 2×10^6 cells were harvested, the resultant pellet was 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 at 50 °C for a further hour. Electrophoresis was carried out in 1.5% agarose gels prepared in TBE buffer (0.45 M tris(hydroxymethyl)aminomethane, 0.45 M boric acid, and 2 mM EDTA, pH 8), at 3 V/cm. A 100−1500 bp DNA standard (Promega) was used to assess DNA fragmentation. DNA was visualized under UV light on a transilluminator (312 nm) following staining with ethidium bromide and photographed using an image analysis system.

Determination of Cellular Glutathione Levels. The cellular level of glutathione was measured according to the method previously described in Ryan et al. (*13*). Briefly 4×10^6 cells were lysed by sonication and centrifuged at 100000*g* for 25 min. Supernatant (100 μL) was diluted in 1.8 mL of phosphate−EDTA buffer (0.1 M sodium phosphate, 0.005 M EDTA, pH 8) and mixed with 100 μL of phthaldialdehyde (1 mg/mL). Samples were incubated at 25 °C for 15 min, and the fluorescence was detected at 420 nm following excitation at 350 nm. The glutathione concentration of the samples was determined from a standard curve. Glutathione concentration was expressed relative to the protein content of the samples. Protein was determined by using the bicinchoninic acid (BCA) method (*15*).

Caspase-3 Activity. Caspase-3 activity was analyzed using a caspase-Glo 7 assay kit supplied by Promega (Ireland). Briefly, U937 cells were seeded in the wells of a 96-well plate and exposed to POP in the presence and absence of antioxidants 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 platereader, and the data were expressed as fold increase relative to an untreated control sample.

Bcl-2 Content. The Bcl-2 level in U937 cells exposed to POP for 24 h was quantified using a Bcl-2 ELISA kit (Calbiochem, QIA23). Briefly, 5×10^6 cells were harvested by centrifugation, and the cells were resuspended in buffer (50 mM Tris containing 5 mM EDTA, 0.2 mM PMSF, 1 μg/mL pepstatin, and 0.5 μg/mL leupeptin, pH 7.4). Antigen extraction agent was added to the extracts (20% v/v), and samples were incubated on ice for 30 min, with occasional vortexing. The samples were then centrifuged for 5 min, and the supernatant was removed. The supernatant was added to the wells of the ELISA plate, and the protocol was carried out according to the supplier's instructions. The absorbance was measured at 450 nm using a Tecan Spectrafluor Plus platereader, and the data are expressed as a percentage of the untreated control sample.

Statistics. All data points represent the mean value (±SE) of at least three independent experiments. The data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's post test when appropriate.

RESULTS AND DISCUSSION

The percentage of viable cells following a 24 h incubation with each of the eight oxidized derivatives of stigmasterol was determined in the U937 cell line using the FDA/EtBr staining assay (**Table 1**). Four of the compounds, 7β-OH (**4**), epoxydiol (**5**), diepoxide (**6**), and 22*R*,23*R*-triol (**7**), were found to induce a significant ($P < 0.05$) level of cell death as compared to the untreated control. 22*R*,23*R*-Triol (**7**) and diepoxide (**6**) caused a significant reduction ($P < 0.05$) in cell viability at each of the three concentrations employed (30, 60, and 120 μM), reducing cell viability to 52.3 and 7.6%, respectively, at the highest concentration (**Table 1**). The epoxydiol (**5**) and 7β-OH (**4**) significantly reduced ($P < 0.05$) cell viability at the 60 and 120 μM concentrations. Three of the four stigmasterol oxides identified as the most cytotoxic were those with an oxidation on the side chain, epoxydiol (**5**), diepoxide (**6**), and 22*R*,23*R*-triol (**7**).

Table 1. Percent Viable U937 Cells Following a 24 h Exposure to 30, 60, or 120 μM Stigmasterol Oxide, As Determined by the Fluorescein Diacetate/Ethidium Bromide (FDA/EtBr) Assay[a]

	30 μM	60 μM	120 μM
α-epoxide	96.8 ± 1.7	96.0 ± 1.5	94.0 ± 0.6
β-epoxide	98.0 ± 1.5	96.3 ± 1.4	96.3 ± 0.9
diepoxide	51.4 ± 8.3*	39.6 ± 4.6*	7.6 ± 2.2*
epoxydiol	95.4 ± 1.5	23.3 ± 3.4*	3.6 ± 0.7*
7-keto	95.7 ± 2.0	96.7 ± 0.7	93.7 ± 1.8
7β-OH	85.8 ± 4.0	56.3 ± 6.5*	22.8 ± 0.6*
3,5,6-triol	97.7 ± 0.6	95.0 ± 1.5	92.3 ± 3.5
22*R*,23*R*-triol	75.0 ± 2.8*	63.3 ± 13.0*	52.3 ± 4.1*

[a] Cell viability in untreated control cells was 97.1 ± 1.0%. Data represent the mean of three individual experiments ± SE. *, $P < 0.05$, ANOVA followed by Dunnetts.

Similar to the α-epoxide and β-epoxide derivatives of β-sitosterol, which previously demonstrated only a marginal cytotoxic effect in the U937 cell line (*8*), the α- (**1**) and β-epoxide (**2**) derivatives of stigmasterol did not have any cytotoxic effects as measured by FDA/EtBr staining. The 7β-OH (**4**) and 22*R*, 23*R*-triol (**7**) derivatives of stigmasterol yielded results comparable to those previously obtained for the equivalent β-sitosterol derivatives, although the 7β-OH stigmasterol (**4**) was more cytotoxic. In contrast, whereas 7-ketositosterol was significantly ($P < 0.05$) cytotoxic (*8*), we did not observe any cytotoxic effects for the 7-ketostigmasterol (**3**) in the present study. A study conducted by Koschutnig et al. (*16*) found that the 7-keto derivative of β-sitosterol was the most cytotoxic POP in the HepG2 cell line followed by 7α-OH, whereas the 7β-OH derivative did not demonstrate any cytotoxic effects. A negative correlation between the cellular uptake of the oxides and their cytotoxic potency was also observed in the study. As stated by the authors, the lack of cytotoxicity observed for 7β-OH-sitosterol in HepG2 cells may be due to cell-specific effects that have previously been demonstrated for COP (*17*).

The cytotoxicity of each of the compounds employed in the present study was also tested using the MTT assay, which measures the metabolic activity of a cell sample. All data are expressed as a percentage of the untreated control sample (**Figure 2**). Unlike the FDA/EtBr staining method, which quantifies the number of live cells in a population, the MTT assay reflects cell proliferation and metabolic activity, which may be increased or decreased under various conditions. 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. All of the oxides except the 3,5,6-triol stigmasterol (**8**) significantly reduced ($P < 0.05$) the metabolic activity of the U937 cells at each of the concentrations employed.

The mechanism of cell death induced by COP in certain cell lines has been identified as apoptosis (*17*, *18*, *19*), whereas a limited number of studies have also shown that β-sitosterol oxides induce apoptosis in the U937 cell line (*6*, *8*). Each of the oxides derived from stigmasterol was tested for its ability to induce apoptosis by a morphological examination of the cell nuclei

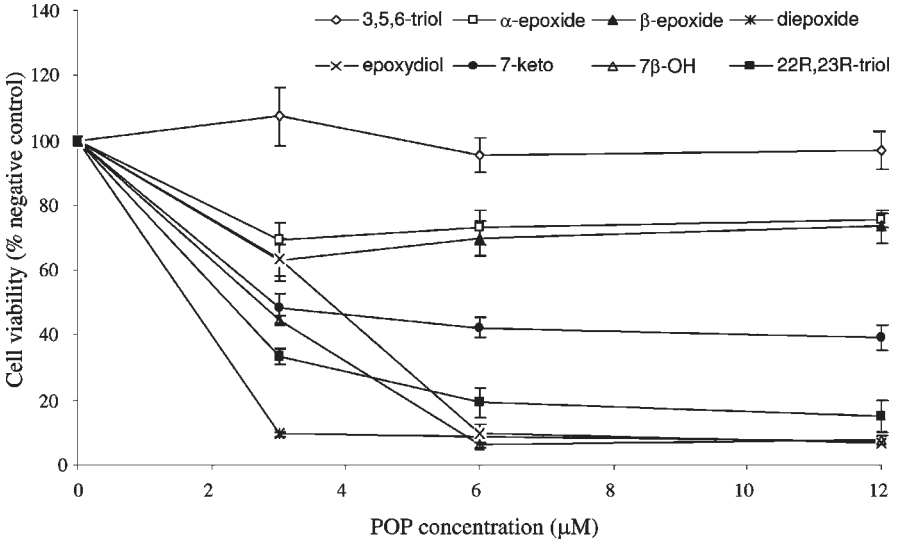


Figure 2. Viability of U937 cells exposed to 30, 60, or 120 μM POP for 24 h as measured by the MTT assay. Data represent four individual experiments ± SE.

Table 2. Percent Apoptotic Nuclei Following a 24 h Incubation with 30, 60, or 120 μM Stigmasterol Oxides, Determined Following Staining with Hoechst 33342[a]

	30 μM	60 μM	120 μM
α-epoxide	2.6 ± 0.2	4.1 ± 1.4	4.4 ± 0.9
β-epoxide	2.9 ± 1.1	3.6 ± 1.1	4.8 ± 0.1
diepoxide	43.9 ± 2.5*	36.8 ± 4.3*	21.6 ± 2.4*
epoxydiol	6.4 ± 0.9	29.3 ± 3.0*	25.3 ± 5.0*
7-keto	5.1 ± 0.6	3.5 ± 0.2	3.9 ± 0.4
7β-OH	9.6 ± 1.1*	20.3 ± 2.2*	19.8 ± 3.8*
3,5,6-triol	2.6 ± 0.3	2.7 ± 1.2	3.0 ± 0.9
22*R*,23*R*-triol	1.7 ± 0.9	2.7 ± 0.6	3.0 ± 0.5

[a] The percent apoptotic nuclei in untreated, control cells was 2.0 ± 0.3%. Data represent the mean of three individual experiments ± SE. *, $P < 0.05$, ANOVA followed by Dunnetts.

following staining with Hoechst 33342. In apoptotic nuclei the DNA is cleaved into regular-sized fragments that give a distinctive appearance and allow the quantification of apoptotic cells in a sample. The level of apoptosis in the control cells did not exceed 2%. Three of the stigmasterol oxides tested induced a significant increase in apoptotic nuclei; epoxydiol (**5**) induced apoptosis at concentrations of 60 and 120 μM, whereas both 7β-OH (**4**) and diepoxide (**6**) caused a significant increase ($P < 0.05$) in the level of apoptotic cells at all of the concentrations tested, 30, 60, and 120 μM (**Table 2**). Increasing concentrations of stigmasterol oxide did not correlate with an increase in apoptosis in all samples, and in cells exposed to diepoxide (**6**) the level of apoptosis was highest at the lowest concentration, indicating that at higher concentrations cells are more likely to die by necrosis as previously observed by Koschutnig et al. (*16*) and Leonarduzzi et al. (*20*). In contrast to the findings for the 7-keto derivative of both cholesterol and β-sitosterol published in Ryan et al. (*8*), we did not find any evidence of apoptosis for the 7-keto (**3**) derivative of stigmasterol. Also, the β-epoxide (**2**) and 22*R*,23*R*-triol (**7**) derivatives of stigmasterol did not induce apoptosis, whereas levels of 7 and 9.5% were detected at the 120 μM concentration for 7-keto and triol β-sitosterol derivatives, respectively (*8*). Studies indicate that the biophysical effects of oxysterols in membrane model systems are governed by their structure, and these membrane effects may be correlated with other biological effects including apoptosis (*21*). It is possible that structural differences between the 7-keto and β-epoxide derivatives of β-sitosterol and the same derivatives of stigmasterol may account

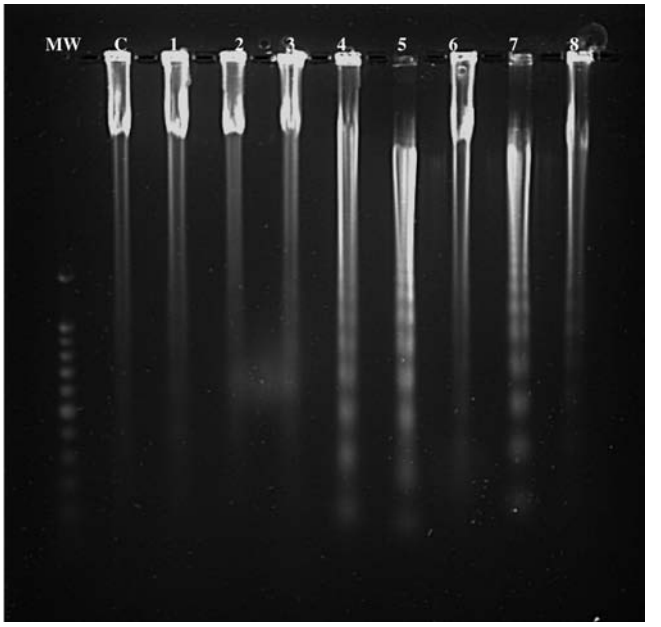


Figure 3. DNA fragmentation in U937 cells exposed to 60 μM POP for 24 h. Lanes: MW, molecular weight marker (200 bp); C control, untreated cells; 1, 3,5,6-triol; 2, α-epoxide; 3, β-epoxide; 4, diepoxide; 5, epoxydiol; 6, 7-keto; 7, 7β-OH; 8, 22*R*,23*R*-triol.

for the differences in the cytotoxicity of the compounds. The 7β-OH derivative of both β-sitosterol (*8*) and stigmasterol (**Table 2**) induced apoptosis, but the effect was more evident for 7β-OH stigmasterol (**4**). The highest levels of apoptosis were induced by the diepoxide (**6**) and epoxydiol (**5**) derivatives, 43.9% at the 30 μM concentration of diepoxide (**6**) and 29.3% at the 60 μM concentration of epoxydiol (**5**), both of which are oxidized on the side chain. FDA/EtBr analysis showed that at 30 μM diepoxide there was 51.4% cell death (**Table 1**), which indicates that at this concentration cell death occurred almost exclusively by apoptosis. 22*R*,23*R*-Triol (**7**) stigmasterol was not found to induce apoptosis in the U937 cell line, and the mode of cell death induced by this oxide was primarily necrosis.

The apoptosis induced by epoxydiol (**5**), diepoxide (**6**), and 7β-OH (**4**)-stigmasterol was confirmed by DNA laddering, where a ladder-like pattern was observed for these samples (**Figure 3**).

Table 3. Percent Apoptotic Nuclei Following 24 h of Incubation with 60 μM Stigmasterol Oxides in the Presence and Absence of α-Tocopherol (10 μM), γ-Tocopherol (10 μM), and β-Carotene (2 μM), Determined Following Staining with Hoechst 33342[a]

	control	α-tocopherol	γ-tocopherol	β-carotene
diepoxide (60 μM)	36.8 ± 4.3	35.2 ± 2.2	33.7 ± 2.2	35.1 ± 4.5
epoxydiol (60 μM)	29.3 ± 3.0	18.6 ± 4.4*	23.2 ± 5.2	28.8 ± 6.3
7β-OH (60 μM)	20.3 ± 2.2	19.0 ± 3.2	23.6 ± 2.6	21.7 ± 4.3

[a] The percent apoptotic nuclei in untreated control cells was 2.0 ± 0.3%. Data represent the mean of three individual experiments ± SE. *, $P < 0.05$, ANOVA followed by Dunnetts.

In the study conducted by Koschutnig et al. (*16*) in the HepG2 cell line, apoptosis was detected only in cells that were exposed to the 7-keto derivative of β-sitosterol and not the 7β-OH and 7α-OH derivatives; however, unlike macrophage cell lines such as U937, HepG2 cells do not generally undergo apoptosis in the presence of COP (*17*).

Several studies have proposed a role for oxidative stress in COP-induced cell death (*13*, *22*), and, recently, data have begun to emerge indicating that POP toxicity proceeds via the generation of superoxide anion and lipid peroxidation (*16*). Also, several studies have indicated that antioxidants including tocopherols and carotenoids may ameliorate the cytotoxic effects of COP (*23*−*25*) and POP (*6*, *8*). Therefore, the effect of antioxidants, α-tocopherol, γ-tocopherol, and β-carotene, on the cytotoxicity and apoptogenicity of cytotoxic stigmasterol oxides, epoxydiol (**5**), diepoxide (**6**), and 7β-OH (**4**) was assessed (**Table 3**). Morphological examination of the cell nuclei did not reveal a protective effect against 60 μM diepoxide (**6**) and 7β-OH (**4**); however, α-tocopherol protected against apoptosis in the epoxydiol (**5**)-treated cells, indicating that the apoptotic pathway induced by epoxydiol (**5**) may differ from that induced by diepoxide (**6**) and 7β-OH (**4**). Vejux et al. (*24*) demonstrated that vitamin E protected against 7-ketocholesterol-induced apoptosis by preventing the inhibition of phosphoinositide 3-kinase (PI3-K) activity and phospholipidosis. It is possible that epoxydiol induces apoptotis via the same mechanism as 7-ketocholesterol; however, further investigation is required to establish the apoptotic pathway invoked by the phytosterol oxides. Lyons et al. (*23*) reported that α-tocopherol but not γ-tocopherol protected against apoptosis induced by 7β-OH cholesterol in U937 cells. Ryan et al. (*8*) found that antioxidants did not protect against the apoptotic effects of 7β-OH-sitosterol and 7-ketositosterol.

Apoptosis may also be quantified by measuring the activity of the apoptotic enzyme caspase-3. The majority of apoptotic signaling pathways converge at caspase-3, which is responsible for the amplification of the apoptotic signal and the activation of downstream enzymes such as PARP, which results in DNA cleavage and cell death (*26*). The integral role of caspase-3 in COP-induced apoptosis was established in a study that observed a suppression of 7β-OH- and β-epoxide-induced apoptosis in the presence of caspase-3 inhibitors (*27*). In the present study caspase-3 activity was increased 3-, 6-, and 7-fold relative to control cells in response to an incubation with 60 μM diepoxide (**6**), epoxydiol (**5**), and 7β-OH (**4**), respectively (**Table 4**). The measurement of caspase-3 activity confirmed that α-tocopherol significantly ($P < 0.05$) protected against epoxydiol-induced apoptosis; a protective effect was also detected for γ-tocopherol and β-carotene. A previous study has also demonstrated that the carotenoid, lycopene, inhibited 7-ketocholesterol-induced apoptosis by limiting caspase-3 activity and the modulatory effects of Bcl-2 in the THP-1 macrophage cell line (*25*).

COP-induced apoptosis is generally initiated by the induction of oxidative stress (*28*). Leonarduzzi et al. (*22*) found that the generation of reactive oxygen species (ROS) was an early event in cells exposed to 7-ketocholesterol and leads to apoptotic cell death. In the study conducted by Koschutnig et al. (*16*), β-sitosterol oxides caused a significant increase in superoxide anion production, whereas the 7-keto and 7α-OH derivatives also caused an increase in lipid peroxidation. The measurement of cellular antioxidant enzymes superoxide dismutase (SOD) and glutathione may also be used to assess the oxidative status of cells exposed to COP or POP. Glutathione has previously been shown to be depleted in the early stages of COP-induced apoptosis (*13*, *28*). At the 60 μM concentration, 7β-OH (**4**), epoxydiol (**5**), and diepoxide (**6**) stigmasterol significantly reduced ($P < 0.05$) the glutathione content of the U937 cells, with epoxydiol (**5**) having the most marked effect (**Table 5**). Glutathione depletion was also a feature of 7β-OH-sitosterol-induced apoptosis (*8*).

Table 4. Caspase-3 Activity (Fold Increase Relative to the Control) in U937 Cells Following 24 h of Incubation with 60 μM Stigmasterol Oxides in the Presence and Absence of α-Tocopherol (10 μM), γ-Tocopherol (10 μM), and β-Carotene (2 μM)[a]

	control	α-tocopherol	γ-tocopherol	β-carotene
diepoxide (60 μM)	3.1 ± 0.1	3.3 ± 0.3	3.7 ± 0.2	2.6 ± 0.2
epoxydiol (60 μM)	6.1 ± 0.1*	4.4 ± 0.1*	4.4 ± 0.1*	4.8 ± 0.1*
7β-OH (60 μM)	7.3 ± 0.4	6.8 ± 0.7	7.9 ± 0.5	6.2 ± 0.1

[a] Data represent the mean of three individual experiments ± SE. *, $P < 0.05$, ANOVA followed by Dunnetts.

Table 5. Glutathione Content and Bcl-2 Content of U937 Cells Following 24 h of Exposure to 60 μM Epoxydiol, Diepoxide, and 7β-OH[a]

	GSH content (nmol/mg of protein)	Bcl-2 (% of control)
control	16.0 ± 0.3	100
epoxydiol	4.0 ± 0.2*	69.0 ± 15.4
diepoxide	5.8 ± 0.5*	50.7 ± 2.9*
7β-OH	10.3 ± 0.4*	59.3 ± 6.3*

[a] Data represent the mean of three individual experiments ± SE. *, $P < 0.05$, ANOVA followed by Dunnetts.

Bcl-2 proteins are involved in the regulation of apoptosis at the mitochondrial level. One of the mechanisms employed by Bcl-2 in the manipulation of the apoptotic process involves an increase in intracellular glutathione, which has been demonstrated to inhibit apoptotic cell death (*29*). The involvement of Bcl-2 in COP-induced apoptosis has previously been demonstrated; however, it was also observed that COP-induced apoptosis can proceed via a mechanism that does not involve Bcl-2, and not all COP operate by the same mechanisms at the mitochondrial level (*30*). The data obtained in the present study (**Table 5**) indicate that there was a down-regulation of Bcl-2 in the presence of apoptotic stigmasterol oxides; both diepoxide (**6**) and 7β-OH (**4**) stigmasterol significantly ($P < 0.05$) down-regulated Bcl-2 relative to an untreated control.

In conclusion, the stigmasterol oxides 7β-OH (**4**), epoxydiol (**5**), diepoxide (**6**), and 22,23*R*-triol (**7**) were cytotoxic to the U937 cell line, and the 7β-OH (**4**), epoxydiol (**5**), and diepoxide (**6**) derivatives were found to induce apoptosis. The diepoxide (**6**) and epoxydiol (**5**) 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, consequently, the side-chain oxide POP warrant further investigation. At 30 μM diepoxide (**6**), the mode of cell death induced was almost exclusively apoptotic. However, the concentrations of phytosterol oxides employed in the current study are in excess of those reported to have been measured in human plasma and are reflective of pharmaceutical rather than physiological levels. The pathway of apoptosis invoked by stigmasterol oxides involved glutathione depletion, caspase-3 activation, and Bcl-2 down-regulation; however,

further investigation is necessary to fully elucidate the pathway of apoptosis induced by these compounds.

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