Pergamon

PII: S0031-9422(96)00818-7

Phytochemistry, Vol. 45, No. 2, pp. 297–302, 1997

0031 9422/97 $17.00+0.00

OXIDIZED PHYTOSTEROLS INCREASE BY AGEING IN PHOTOAUTOTROPHIC CELL CULTURES OF *CHENOPODIUM RUBRUM*

WERNER MEYER and GERHARD SPITELLER*

Lehrstuhl für Organische Chemie I, Universität Bayreuth, NWI, Universitatsstrasse 30, Bayreuth, Germany

(*Received* 27 *June* 1996)

Key Word Index—*Chenopodium rubrum*; Chenopodiaceae; photoautotrophic suspension cell culture; phytosterols; sitosterol; campesterol; stigmasterol; phytosterol epoxides; 3,5,6-trihydroxyphytosteranes; age related changes.

Abstract—Young and old cell cultures of *Chenopodium rubrum* were investigated for their content of phytosterol derivatives by gas chromatography–mass spectrometry. The ratio of the main phytosterol constituents, sitosterol, stigmasterol and campesterol (1.2 : 2.4 : 1.0) remained approximately constant, but their overall content (402.5 $\mu g\ g^{-1}$ dry wt) decreased from 11 days to 8 weeks old cell cultures by about 64% (260.1 $\mu g\ g^{-1}$ dry wt); in contrast the content of corresponding epoxides increased from traces at day 11 to 11.1 $\mu g\ g^{-1}$ (dry wt) at the 8th week. A comparable increase was observed for 3,5,6-trihydroxysteranes (2.8 $\mu g\ g^{-1}$ dry wt), day 11; 27.5 $\mu g\ g^{-1}$ dry wt), 8 weeks). The content of sterols oxidized in position C-7 showed also an increase from 1.9 $\mu g\ g^{-1}$ dry wt) (11 days) to 51.7 $\mu g\ g^{-1}$ dry wt) (8 weeks). These results show that the increase of phytosterol oxidation products correlated with age of the cell culture. Therefore, the sterol epoxide content and related oxidation products may be used as a measure for ageing.

INTRODUCTION

Phytosterols are membrane constituents of all plants. They are important in controlling the fluidity of the membrane by stabilizing the lipid bilayer [1]. Phytosterols as campesterol **2**, sitosterol **3** and stigmasterol **4** contain a Δ^5-double bond, which can be epoxidized by lipid peroxides as reported for cholesterol [2, 3].

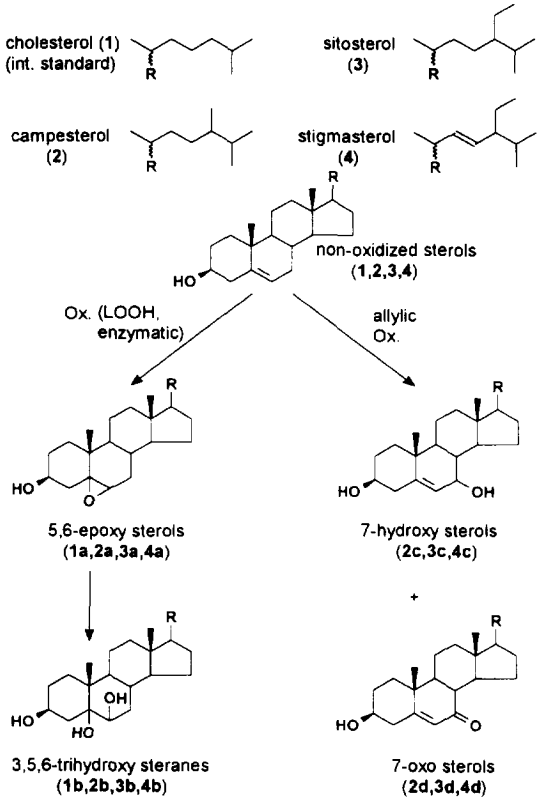
Recently we observed that the content of caryophyllene oxide (the epoxide of caryophyllene) is increased threefold in aged lemon balm leaves, compared to young ones; obviously in correlation with an increase in lipid peroxidation [4]. Epoxidation by LOOH seems to be a common reaction which involves all types of double bonds [5]. Since LOOH are assumed to be produced in increased amounts in ageing [6], we suspect that epoxidation might be a process connected with ageing. Therefore, we investigated whether the content of sterol epoxides also increases in aged plants.

RESULTS

It is difficult to determine the age related changes in oxidation products of plants, because oxidative processes seem to be influenced by external factors e.g. habitat [4]. In addition, age determination of cells in plant leaves is difficult because of the different age of differentiated cells in tissue. Therefore, we decided to use photoautotrophic suspension cell cultures which are a suitable model system for green plants, because of their ability to photosynthesize. Furthermore their proliferation can be synchronized by a constant light–dark-rhythm [7]. Thus, this cell culture produces homogeneous undifferentiated cell material of defined age.

Suspension cell cultures of *Chenopodium rubrum* were grown under controlled conditions [7] (see Experimental) and were harvested after 11 days (linear growth), 4 weeks (stationary phase) and 8 weeks (end of stationary phase, begin of cell death). After removal of the growth medium the cells were broken by ultrasonic treatment in methanol–methylene dichloride and internal standards were added. In previous workups we found that cholesterol **1** and oxidation products thereof are detected in trace amounts only. Therefore, cholesterol (**1**), 5,6-epoxy-cholesterol (**1a**) and 3,5,6-trihydroxy-cholestane (**1b**) were used as internal standards. BHT was added to prevent autoxidation during the workup procedure. By Bligh–Dyer extraction [8] the lipids were obtained. Hydroperoxides were reduced to corresponding hydroxy derivatives by

*Author to whom correspondence should be addressed.



Scheme 1. Main phytosterol constituents of photoautotrophic suspension cell cultures of *Chenopodium rubrum* and the detected and quantified oxidation products.

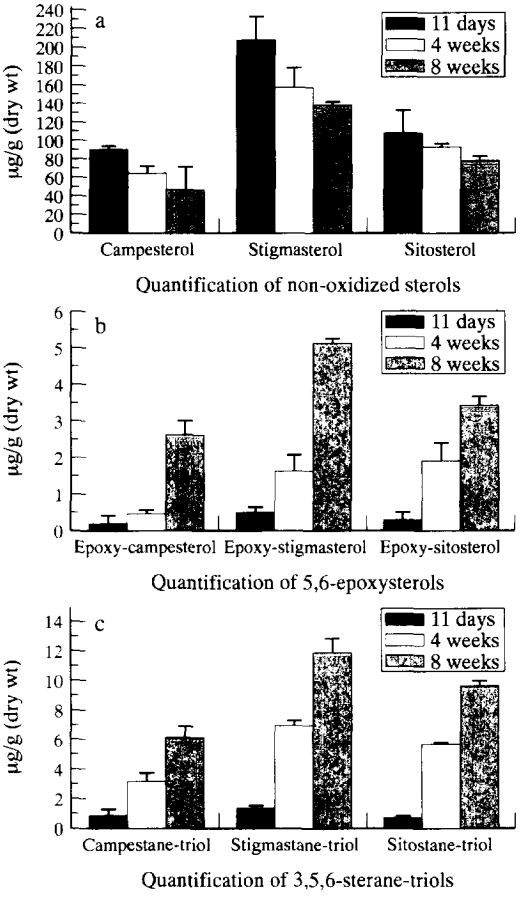


Fig. 1. Content of phytosterols (A), 5,6-epoxy sterols (B) and 5,6-dihydroxy derivatives (C) of *Chenopodium rubrum* cell culture harvested after 11 days, 4 weeks and 8 weeks.

sodium borohydride treatment. After hydrolysis of lipids and steryl esters, followed by methylation of fatty acids with diazomethane, oxidized sterols were separated from less polar sterols and lipids by preparative TLC. The TLC fractions containing sterols were trimethylsilylated and subjected to GC-mass spectrometry. For quantification of 7-oxidized sterols 7-oxositosterol (**3d**) and 7α-hydroxysitosterol (**3c**) were added to one sample of the TLC fraction B and this was injected into GC-mass spectrometry after trimethylsilylation. Comparison to a similar treated sample, but without addition of standards, enabled quantification. All measurements were carried out three times to determine the experimental errors. The phytosterols and derived oxidation products were identified by GC retention indices and by mass spectra which showed molecular ions and typical cleavage fragments [9–12].

The main phytosterol constituents of the suspension cell culture of *Chenopodium rubrum* were campesterol (**2**), sitosterol (**3**) and stigmasterol (**4**). The main oxidation products detected were the 5,6-epoxides (**2a**, **3a**, **4a**) and 3,5,6-trihydroxysteranes (**2b**, **3b**, **4b**), as well as 7-hydroxysterols (**2c**, **3c**, **4c**) and 7-oxosterols (**2d**, **3d**, **4d**) (Scheme 1).

Quantification of non-oxidized phytosterols (**2**, **3**, **4**) was achieved by GC with the internal standard cholesterol (**1**) because the response factors of the sterols **2**–**4** in relation to **1** were $f_i \approx 1$. Due to the low amounts of oxidized phytosterols no GC integration of these compounds could be achieved. Therefore, oxidized phytosterols (as TMSi-derivatives) were quantified by reference to specific ions in the reconstructed ion chromatogram (RIC) after GC-mass spectrometry analysis. Quantification of 5,6-epoxysterols (**2a**, **3a**, **4a**) was done by comparison of the intensities of the ions $[M^+]$ (~50% rel. int.) in relation to the internal standard (5,6-epoxycholesterol **1a**). In the case of 3,5,6-trihydroxy phytosteranes (**2b**, **3b**, **4b**) the ions $[M^+ - 90]$ (~55% rel. int.), $[M^+ - 2 \times 90]$ (100% rel. int.), $[M^+ - 3 \times 90]$ (~40% rel. int.) were used for quantification (internal standard: 3,5,6-trihydroxy cholestane, **1b**).

The non-oxidized sterols (**2**, **3**, **4**) were quantified by using the ions $[M^+$, rel. int. ~40%] and $[M^+ - 90$, rel. int. 100%] in order to compare the results of the ion trace method with GC quantification. Comparable absolute amounts were measured. The results of these investigations are represented in Fig. 1.

The ratio of the main phytosterol constituents, sito-

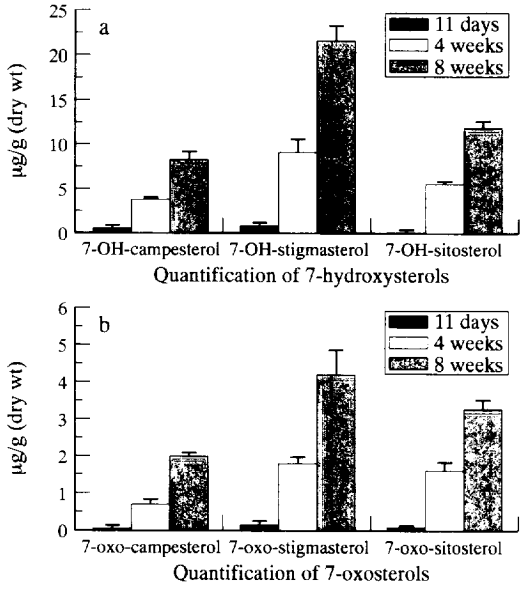


Fig. 2. Content of 7-hydroxy sterols (E) and 7-oxo derivatives (F) of *Chenopodium rubrum* cell culture harvested after 11 days, 4 weeks and 8 weeks.

sterol (**3**), stigmasterol (**4**) and campesterol (**2**) (1.2 : 2.4 : 1.0) remained approximately constant during the growth period, but their overall content (402.5 $\mu g\ g^{-1}$ dry wt) decreased from 11 days to 8 weeks old cell cultures by 64% (260.1 $\mu g\ g^{-1}$ dry wt); in contrast the content of corresponding epoxides (**2a**, **3a**, **4a**) increased from traces (1.0 $\mu g\ g^{-1}$ dry wt) at day 11 to 11.1 $\mu g\ g^{-1}$ dry wt) at the 8th week. The increase of **2a**, **3a**, **4a** is low during the first 4 weeks (17–35% of the content at week 8), but raised strongly between week 4 and 8. The content of 3,5,6 trihydroxysteranes **2b**, **3b**, **4b** increased linearly from 2.8 $\mu g\ g^{-1}$ (dry wt) at day 11 to 27.5 $\mu g\ g^{-1}$ (dry wt) at week 8.

In addition, 7-oxygenated sterols (allylic oxidation products) were detected: 7-hydroxy-(**2c**, **3c**, **4c**) and 7-oxo-derivatives (**2d**, **3d**, **4d**). Quantification of 7-hydroxyphytosterols (**2c**, **3c**, **4c**) and 7-oxo-phytosterols (**2d**, **3d**, **4d**) was achieved by addition of 7α-hydroxysitosterol (**3c**) and 7-oxositosterol (**3d**) to one sample of TLC fraction B and measurement of the ions $[M^+\text{-}90]$ (100% rel. int.) for quantification of 7-hydroxyphytosterols (**2c**, **3c**, **4c**), respectively, the ions $[M^+]$ (100% rel. int.), $[M^+\text{-}90]$ (~20% rel. int.) for quantification of 7-oxophytosterols (**2d**, **3d**, **4d**). Absolute amounts were obtained by comparison with the relative intensities of those ions in a sample without addition of C-7 oxidized standards (**3c**, **3d**).

The contents of these products were also increased with age (Fig. 2). The C-7 oxidized phytosterols were found only in traces in young cell culture material, but the amounts of 7-oxophytosterols (**2d**, **3d**, **4d**) and 7-hydroxyphytosterols (**2c**, **3c**, **4c**) increased to 9.5 $\mu g\ g^{-1}$ (dry wt) and 42.4 $\mu g\ g^{-1}$ (dry wt), respectively, at the 8th week of growth. The over all content of allylic oxidized phytosterols (**2c**, **3c**, **4c**; **2d**, **3d**, **4d**) raised from 1.9 $\mu g\ g^{-1}$ (dry wt) (11 days) to 51.7 $\mu g\ g^{-1}$ (dry wt) in week 8.

DISCUSSION

The occurrence of phytosterol oxidation products had been reported only occasionally: oxidized sitosterols were isolated from soy bean oil and wheat floor [12]. Phytosterol epoxides were detected after storage of food containing plants [13, 14], or after drying of plant material [15].

In contrast, cholesterol oxidation products were observed in many instances and their genesis had been investigated in detail: 7α- and 7β-hydroxycholesterol, 7-oxocholesterol, the 5,6α- and β-epoxides and the derived corresponding 3,5,6-cholestanetriols were identified in meat products [16]. It was found that these compounds are involved in numerous pathological processes, e.g. cancerogenesis [2, 17] or atherosclerosis [17, 18]. Cholesterol epoxides and especially their hydrolysis products, 3,5,6-cholestanetriols are cytotoxic [17, 19]. Cholesterol epoxides furthermore bind to DNA [20] and react with SH-nucleophiles (e.g. glutathione) [21].

Cholesterol derivatives oxidized at C-7 are generated via allylic oxidation to the isomeric hydroperoxy cholesterols [22]. These were detected in rat skin and used as ageing markers [23]. 7-Hydroperoxysterols are thermically unstable and, therefore, they cannot be analyzed by GC [24, 25]. They suffer decomposition to 7-oxosterols, which have been described as constituents of plant tissues [26, 27]. In contrast to these primary oxidation products the epoxides of cholesterol are produced in a more complicated reaction. The epoxides were obtained either by incubation of cholesterol with linoleic acid/lipoxygenase [28] or linoleic acid hydroperoxide/Fe^{2+} [2], indicating a lipid peroxidation mediated radical mechanism for sterol epoxidation. This was confirmed by the observation that the reaction was inhibited by addition of α-tocopherol or BHT [29]. Otherwise cholesterol epoxidation was discussed to be achieved by reactive oxygen species (ROS: H_2O_2, $HO\cdot$, singlet oxygen, alkoxy- and peroxy radicals) [30, 31].

In order to avoid artificial oxidation by reactive oxygen species or enzymes in the present work the samples were processed in an argon atmosphere, BHT was added as antioxidant and enzymes were inactivated by the extraction solvent (methanol–dichloromethane). Finally oxidation by active hydroperoxides during workup was prevented by reduction to hydroxy products with sodium borohydride. Thus, an artificial generation of oxidation products could be excluded. Furthermore, the amount of detected phytosterol oxidation products increased with age, which demonstrated that these products are *in vivo* plant constituents. Although the content of sterol epoxides and derived hydrolysis products, as well as C-7 oxidized sterols, is low compared to non-

oxidized phytosterols their content seems to reflect the process of ageing.

We assume that the generation of epoxides is enzymatically controlled. Epoxidation *in vivo* is either achieved directly by action of enzymes, e.g. epoxidases or cytochrome P-450 [32], or via an oxidation process after cell injury in which unsaturated fatty acids are transformed to hydroperoxides by lipoxygenases. These react with unsaturated compounds by epoxidation as previously described [2, 5].

Hydroperoxides are generated in destroying processes like cell injury by action of lipoxygenases [33, 34]. Cell destroying processes are increased by ageing, one of the primary ageing theories—the free radical/lipid peroxidation theory [35]—correlates ageing with oxidative stress. An age related increase of lipid peroxidation was reported in ref. [6].

The increase of epoxides during ageing might have physiological consequences. It was demonstrated that ethylene and propylene epoxides react with peptides by opening the epoxide ring to form hydroxyethyl derivatives [36]. In addition epoxides react with SH-nucleophiles which causes a decrease of antioxidative cell protection components, e.g. glutathione, during ageing [37, 38]. Therefore, one must assume that SH containing enzymes may also react in a similar manner. In the course of those reactions enzymes might be deactivated which could promote an increasing enzyme malfunction in ageing cells.

In addition, the oxidation of phytosterols, which are essential plant membrane lipids, may change the membrane permeability and may offer easier access of damaging compounds in the cell in a similar manner as reported for mammalian cells in which cholesterol oxidation products were assumed to cause these effects [39]. Thus, the increase in phytosterol oxidation products, especially epoxides and related compounds, might be used as markers for ageing of plant cells.

EXPERIMENTAL

Materials. *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) was obtained from Macherey and Nagel (Düren). All other chemicals were purchased from Fluka (Neu Ulm). Solvents, obtained from Merck, were distilled before use. TLC was performed on home made 0.75 mm PF_{254} silica gel 60 (Merck) plates.

Plant material. Suspension cell cultures of *Chenopodium rubrum* were grown photoautotrophically under sterile conditions by the method of Hüsemann and Barz [7]. 2 week old starting cultures were obtained from Dr Christian Schäfer (Institute of plant Physiology, University of Bayreuth, Germany).

Cultivation was done in special two-tiered flasks. The lower compartment was filled with 50 ml K_2CO_3 buffer (0.2 M $KHCO_3$: 0.2 M K_2CO_3 3:1 v v) which delivered a CO_2 concentration of approximately 2% [40]. The upper compartment contained 60 ml of MURASHIGE-SKOOG media (MS, Sigma M5519) [41] to which 1% of a 2,4-dichlorophenoxyacetic acid solution (10^{-5} M, 2,4-D) was added. The pH was adjusted to 5.6. Subcultivation was done by transferring 10 ml of 14 days old suspension culture to new MURASHIGE-SKOOG media. Cultivation was performed in a constant light–dark cycle (16 hr light, photon flux density 75 μmol m^{-2} s^{-1}, 28°; 8 hr dark, 25°) by shaking at 105 rpm (TR 125 shaker, Infors). The cells were harvested on a sintered glass filter and carefully rinsed with distilled water. Plant material was frozen in liquid nitrogen. It could be stored at −20° for a few weeks without compositional changes.

Extraction of sterols. The lipid fraction was extracted from plant material according to a modified procedure of Bligh and Dyer [8, 42]: 50 g plant material was suspended in 300 ml MeOH–CH_2Cl_2 (2:1). BHT (0.1%) was added to avoid autoxidation [43]. Homogenization by ultrasonic treatment for 10 min. The following internal standards were added for quantification: cholesterol (2 ml of 100 μg ml^{-1} soln), 5,6-epoxycholesterol (2 ml of 10 μg ml^{-1} soln), 5,6-dihydroxy-cholesterol (2 ml of 10 μg ml^{-1} soln). The soln was stirred in an argon atmosphere for 1 hr, then it was filtered, and after centrifugation (1000 rpm, 4 min) the organic phase was separated from the aq. phase and the solvent removed under vacuum. About 2 g crude lipid extract was obtained. The residue was redissolved in 50 ml MeOH–CH_2Cl_2 (2:1) and 0.2 g $NaBH_4$ was added to prevent further oxidation by hydroperoxides. After stirring for 2 hr at 25° the excess $NaBH_4$ was hydrolyzed with 50 ml H_2O. After extraction with 50 ml CH_2Cl_2 (3×) and removal of solvent the lipid residue (containing steryl esters) was hydrolyzed by stirring with 90 ml THF-sodium hydroxide (1:2) for 3 hr. 100 ml H_2O was added and the soln was acidified with 0.1 N HCl. After extraction with CH_2N_2 (50 ml, 3×) the soln was dried *in vacuo*. The remaining residue (about 500 mg) was redissolved in 20 ml MeOH and methylated by addition of etheral CH_2Cl_2 to yield the fatty and methyl esters. Further separation of oxidized sterols was carried out by prep. TLC (cyclohexane–EtOAc, 1:1). Detection was by spraying with methanolic molybdatophosphoric acid and heating for 5 min and by measuring of UV absorption (254 nm). Three fractions were obtained: (A) non-oxidized phytosterols (R_f = 0.8–0.5, standard: cholesterol R_f = 0.68); (B) epoxy- and monohydroxysterols (R_f = 0.5–0.25, standard: 5,6-epoxycholesterol, R_f = 0.41); (C) higher oxidized sterols (R_f = 0.25–0.05, standard: 5,6-dihydroxy-cholesterol, R_f = 0.1). A 0.3 mg sample of each fraction was trimethylsilylated with 10 μl MSTFA and subjected to GC-MS analysis. All analyses were performed 3×.

GC- and GC-MS analysis. GC was carried out on a United Technologies Packard Model 438S chromatograph equipped with a flame ionisation detector (FID) and a Shimadzu C-R3A integrator. GC conditions: WCOT fused silica DB-1 capillary column (30 m × 0.32 mm i.d., film thickness 0.1 μm, J&W Scientific, Mainz-Kastel); carrier gas: hydrogen; split-

ting ratio: 1 : 10; injector temperature: 270°; detector temperature: 290°; temperature program: 80° isotherm for 3 min, heating rate from 80 to 280°, 3° min^{-1}, 280° isotherm for 15 min; GC injections were carried out twice. Linear retention indices were determined by coinjection of a mixture of *n*-alkanes (C_{10}–C_{40}) [44].

Compound identification was achieved by GC-MS: Gas chromatograph HP 5890 series II, fused silica DB-1 capillary column (30m × 0.32 mm i. d., film thickness 0.1 μm, J&W Scientific, Mainz-Kastel; all other conditions were the same as reported above for GC). The GC was coupled to a Finnigan MAT 95 mass spectrometer, data system MAT ICIS (DEC station 5000/120); ionisation energy: 70 eV.

Quantification. Quantification of non-oxidized phytosterols was done by GC peak area integration. Absolute compound amounts were obtained by comparison with the area of the internal standard cholesterol. The response factor of cholesterol and the phytosterols was determined to be 1 within experimental error. Thus peak area correction was not necessary.

Oxidized phytosterols were quantified by ion tracing of specific ions in the RIC of the MS: epoxysterols (as TMSi-derivatives) were determined by comparison of peak intensities of the ions $[M^+]$, $[M^+-15]$, $[M^+-18]$, $[M^+-90]$ in relation to the intensities of the internal standard (5,6-epoxy-cholesterol). 3,5,6-trihydroxysteranes (as TMSi-derivatives) were determined by comparison of peak intensities of the ions $[M^+-90]$, $[M^+-2\times90]$, $[M^+-3\times90]$ in relation to the intensities of the internal standard (3,5,6-trihydroxy-cholestane). Absolute amounts were calculated by taking the average of the valves determined from the single ions. Quantification of 7-hydroxyphytosterols and 7-oxophytosterols was achieved by addition of 7α-hydroxysitosterol and 7-oxositosterol to one sample of TLC fraction B and measurement of the ions $[M^+-90]$ (100% rel. int.) for 7-hydroxyphytosterols respectively the ions $[M^+]$ (100% rel. int.), $[M^+-90]$ (~20% rel. int.) for 7-oxophytosterols. Absolute amounts were obtained by comparison with the relative intensities of those ions in a sample without addition of C-7 oxidized standards.

Synthesis of internal standards: epoxidation of cholesterol. The method was modified from ref. [45]. Cholesterol (3.0 g, 7.75 mmol) was dissolved in 100 ml CH_2Cl_2 and 50 ml saturated $NaHCO_3$ were added and stirred at 0° *meta*-chloroperbenzoic acid (mCPBA) (1.9 g) was added in small portions during 1 hr. After stirring for another hour the organic phase was separated and the aq. phase was extracted 3× with CH_2Cl_2. The combined organic phases were washed with H_2O, 10% KOH and saturated NaCl. After removal of the solvent, 3.1 g (99.8% yield) of 5,6-epoxy-cholesterol w obtained. The crude product was purified by prep. TLC on silica gel (cyclohexane–EtOAc, 2:1 $R_f = 0.32$, 99.9% purity (GC)). TMSi-derivative: GC (DB-1): RI = 3209. GC-EIMS 70 eV, *m/z* (rel. int.): 474[M^+] (42), 459[M^+-Me] (18), 456[M^+-H_2O] (19), 384[M^+-TMSiOH] (45), 366[M^+-TMSiOH-H_2O] (100), 351[M^+-TMSiOH-H_2O-Me] (28), 253[M^+-TMSiOH-H_2O-113] (26). IR ν_{max} cm^{-1}: 3425 (O-H). ^1H NMR (500 MHz, $CDCl_3$): δ 0.58 (3H, *s*, H-18), 0.83 (6H, *d*, $J = 6.5$ Hz, H-26 and H-27), 0.86 (3H, *d*, $J = 7$ Hz, H-21), 1.04 (3H, *s*, H-19), 1.67 (1H, *dd*, H-4e), 2.05 (1H, *dd*, $J_{4a,4e} = 13$ Hz, $J_{3a,4a} = 11$ Hz, H-4a), 2.88 (1H, *m*, H-6), 3.88 (1H, *m*, H-3a). ^{13}C NMR (125,76 Mhz, $CDCl_3$): δ 11.8 (C-18), 15.9 (C-19), 18.6 (C-21), 59.3 (C-6), 65.7 (C-5), 68.7 (C-3). Spectral data were in agreement with literature data [16, 46, 47].

Synthesis of 3β,5α,6β-trihydroxy-cholestane. Modified from ref. [48] 5,6-Epoxy cholesterol (200 mg, 0.5 mmol) was dissolved in 40 ml THF and 0.5 ml H_2O added. Nafion NR-50-H^+ (Fluka) (500 mg) which was activated for 12 hr at 120° was added and the soln stirred for 12 hr at room temp. After removal of the catalyst and the solvent the product was dried by azeotropic distillation with CH_2Cl_2. The crude product was dissolved in MeOH and purified by prep. TLC (cyclohexane-EtoAc, 1:4). $R_f = 0.19$, 99.4% purity (GC), 90 mg (45% yield) of pure 3,5,6-trihydroxy-cholestane were obtained; 70 mg (35% yield) 5,6-epoxy-cholesterol was recovered.

TMSi-derivative: GC (DB-1): RI = 3405. GC-EIMS 70 eV, *m/z* (rel. int.): 636[M^+] (0), 546[M^+-TMSiOH] (53), 531[M^+-TMSiOH-Me] (29), 456[M^+-2 × TMSiOH] (100), 441[M^+-2 × TMSiOH-Me] (35), 403 (51), 366[M^+-3 × TMSiOH] (72), 351[M^+-3 × TMSiOH-Me] (21), 321 (36), 253[M^+-3 × TMSiOH-113] (21). IR ν_{max} cm^{-1}: 3425 (O-H). ^1H NMR (500 Mhz, CD_3OD): δ 0.71 (3H, *s*, H-18), 0.88 (6H, *d*, $J = 6.5$ Hz, H-26 and H-27), 0.93 (3H, *d*, $J = 7$ Hz, H-21), 1.15 (3H, *s*, H-19), 1.67 (1H, *dd*, $J_{4a,4e} = 13$ Hz, $J_{3a,4e} = 4$ Hz, H-4e), 2.05 (1H, *dd*, $J_{4a,4e} = 13$ Hz, $J_{3a,4a} = 11$ Hz, H-4a), 3.44 (1H, *m*, H-6), 3.99 (1H, *m*, H-3a). ^{13}C NMR (125,76 Mhz, CD_3OD): δ 12.6 (C-18), 17.3 (C-19), 68.3 (C-3), 76.5 (C-6), 76.8 (C-5). Spectral data were in agreement with literature data [16, 47, 49].

Acknowledgements—We thank Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie for financial support. We are obliged to Mr M. Glaessner for running the mass spectra and Dr J. Reiner for NMR measurements. We are grateful to the group of PD Dr C. Schäfer for their assistance with the cell culture. We are thankful to Prof. K. Seifert for providing us samples of 7-hydroxy- and 7-oxo-sitosterol.

REFERENCES

1. Leshem, Y., Shewfelt, R. and Willmer, C., in *Plantmembranes*. Kluwer Academic Press, London, 1993, pp. 56–61.
2. Watabe, T., Tsubaki, A., Isobe, M., Ozawa, N.

and Hiratsuka, A., *Biochimica et Biophysica Acta*, 1984, **795**(1), 60.
3. Muto, T., Tanaka, J., Miura, T. and Kimura, M., *Chemical and Pharmaceutical Bulletin*, 1982, **30**(9), 3172.
4. Meyer, W. and Spiteller, G., *Zeitschrift für Naturforschung. C*; 1996, **51c**, 651.
5. Meyer, W. and Spiteller, G., *Liebigs Annalen der Chemie*, 1993, 1253.
6. Dhindsa, R. S., Plumb-Dhindsa, P. and Thorpe, T. A., *Journal of Experimental Botany*, 1981, **32**, 93.
7. Hüsemann, W. and Barz, W., *Plant Physiology*, 1977, **40**, 77.
8. Bligh, E. G. and Dyer, W. J., *Canadian Journal of Biochemistry and Physiology*, 1959, **37**, 911.
9. Smith, A. G. and Brooks, C. J. W., *Biomedical Mass Spectrometry*, 1976, **3**, 81.
10. Aringer, L. and Nordstroem, L. *Biomedical Mass Spectrometry*, 1981, **8**, 183.
11. Blekas, G., Boskou, D., *Food Chemistry*, 1989, **33**, 301.
12. Nourooz-Zadeh, J. and Appelqvist, L., *Journal of the American Oil Chemists Society*, 1992, **69**, 288.
13. Lee, K., Herian, A. M. and Richardson, T. *Journal of Food Protection*, 1984 **47**, 340.
14. Lee, K., Herian, A. M. and Higley, N. A., *Journal of Food Protection*, 1985, **48**, 158.
15. Della Greca, M., Fiorentino, A., Molinaro, A., Monaco, P. and Previtera, L., *Natural Products Letters*, 1993, **2**, 27.
16. Park, S. W. and Addis, P. B., *Journal of Food Science*, 1987, **52**, 1500.
17. Smith, L. L. and Johnson, B. H., *Free Radical Biology and Medicine*, 1989, **7**, 285.
18. Peng, S. K., Hu, B. and Morin, R. J., *Journal of Clinical Laboratory Analysis*, 1991, **5**, 144.
19. Peng, S. K., Taylor, C. B., Hill, B., Morin, J. C. and Robert, J., *Atherosclerosis* (*Shannon, Irel.*), 1985, **54**, 121.
20. Blackburn, G. M., Rashid, A. and Thompson, M. H., *Journal of the Chemistry Society Chemistry Communications*, 1979, **9**, 420.
21. Pickett, C. B. and Lu, A. Y. H., *Annual Review of Biochemistry*, 1989, **58**, 743.
22. Smith, L. L. in *Autoxidation in Food and Biological Systems* ed. M. G. Simic and M. Karel. Plenum Press, London, 1980, p. 119.
23. Ozawa, N., Yamazaki, S., Chiba, K., Aoyama, H., Tomisawa, H., Tateishi, M. and Watabe, T., *Biochemical and Biophysical Research Communications*, 1991, **178**(1), 242.
24. Sevanian, A. and McLeod, L. L., *Lipids*, 1987, **22**, 627.
25. Sevanian, A., Seraglia, R. and Traldi, P., *Free Radicals in Biology and Medicine*, 1994, **17**, 397.
26. Schroeder, G., Rohmer, M., Beck, J. P. and Anton, R., *Phytochemistry*, 1980, **19**, 2213.
27. Chaurasia, N. and Wichtl, M., *Journal of Natural Products*, 1987, **50**, 881.
28. Aringer, L., *Lipids*, 1980, **15**, 563.
29. Johannson, G., *European Journal of Biochemistry*, 1971, **21**, 68.
30. Yanishlieva, N., Marinova, E., Schiller, H. and Seher, A., *Developments in Food Science*, 11(*Fat Science* 1983, *Pt B*), 1985, 619.
31. Gumulka, J., Pyrek, J. S. and Smith, L. L., *Lipids*, 1982, **17**, 197.
32. Capdevila, J. H., Dishman, E., Karara, A. and Falck, J. R., *Methods in Enzymology*, 206 (*Cytochrome P*450), 1991, 441.
33. Dargel, R., *Experimental Toxicology and Pathology*, 1992, **44**, 169.
34. Spiteller, G., *Journal of Lipid Mediators*, 1993, **7**, 199.
35. Bland, J., *Journal of Chemistry Education*, 1978, **55**, 151.
36. Toernqvist, M., *Carcinogenesis* (*London*), 1990, **11**, 51.
37. Tsikas, D. and Brunner, G., *Fresenius' Journal of Analytical Chemistry*, 1992, **343**, 330.
38. Silva, M. H., Wixtrom, R. N. and Hammock, B. D., *Cancer Research*, 1988, **48**, 1390.
39. Ursini, F., Maiorino, M. and Sevanian, A., in *Oxidative Stress*, ed. H. Sies, Academic Press, London, 1991, pp. 319.
40. Schäfer, C. and Schmidt, E., *Physiologia Plantarum*, 1991, **82**, 440.
41. Murashigge, T. and Skoog, F., *Physiologia Plantarum*, 1962, **15**, 473.
42. Kates M., in: *Laboratory Techniques in Biochemistry and Molecular Biology*, Vol. 3, Part 2, eds. R. H. Burdon and P. H. Van Knippenberg. Elsevier, Amsterdam, 1986, pp. 104.
43. Esterbauer, H. and Cheeseman, K., *Methods in Enzymology*, 1990, **186**, 407.
44. Van den Dool, H. and Kratz, P. D., *Journal of Chromatography*, 1963, **11**, 463.
45. Kametani T., Nemoto H. and Fukumoto K., *Bioorganic Chemistry*, 1978, **7**, 215.
46. Ansari, G. A. S. and Smith, L. L., *Methods in Enzymology*, 186(*Oxygen Radicals Biological Systems, Pt B*), 1990, 438.
47. Blunt, J. W. and Stothers, J. B., *Organic Magnetic Resonance*, 1977, **9**, 439.
48. Olah, G. A., Fung, A. P. and Meidar, D., *Synthesis*, 1981, (4) 280.
49. Kirk, D. N., Toms, H. C., Douglas, C., White, K. A., Smith, K. E., Latif, S. and Hubbard, R. W. P., *Journal of the Chemistry Society, Perkin Transactions* 2, 1990, 1567.