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ON THE CYTOTOXITY OF OXIDIZED PHYTOSTEROLS ISOLATED FROM PHOTOAUTOTROPHIC CELL CULTURES OF *CHENOPODIUM RUBRUM* TESTED ON MEAL-WORMS *TENEBRIO MOLITOR*

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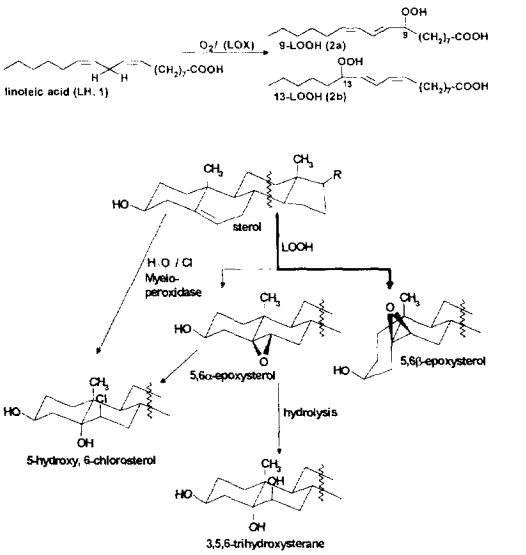
Key Word Index—*Chenopodium rubrum*; Chenopodiaceae; *Tenebrio molitor*; Tenebrionidae; phytosterols; phytosterol epoxides; 3,5,6-trihydroxy-phytosteranes; cytotoxicological testing; bioactivity

Abstract—Cell injury—as evoked by oxidative stress, insect attack or ageing—causes lipid peroxidation of polyunsaturated acids, e.g. linoleic acid. Hydroperoxides (LOOH) so produced transform fatty acids, terpenes and sterols to epoxides. We describe a bioassay to determine the cytotoxity of sterol oxidation products by measuring the mortality of meal-worms (*Tenebrio molitor*) after injection of diluted test compounds. LOOH was tested in concentrations from 10^{-5} M up to 10^{-2} M and developed recognizable mortality (25%, 10^{-2} M, 23 hr). A direct relation between activity and concentration was observed. 5,6-Epoxides of phytosterols and derived 3,5,6-trihydroxysteranes have been isolated from *Chenopodium rubrum* cell cultures. Using 5,6-epoxycholesterol and 3,5,6-trihydroxycholestane as standards of known bioactivity, the toxicity of epoxides of sitosterol and stigmasterol, as well as the trihydroxy compounds thereof, was checked. The phytosterol derivatives are by a factor of five less active than the corresponding cholesterol oxidation products. 5,6-Epoxides and 5,6-chlorohydrins showed half of the activity of the corresponding 3,5,6-trihydroxyphytosterols. These developed highest cytotoxity (40% mortality in 5×10^{-3} M solution after 23 hr). The activity of 5,6α- and 5,6β-epoxides was found to be equal. Metabolism and proposed biological function of oxidized phytosterols is discussed.

INTRODUCTION

Plant cell injury, for instance by oxidative stress, insect attack or ageing, causes enzymatic hydrolysis of membrane lipids [1, 2]. Thus hydrolysed linoleic acid (**1**) is oxidized in the presence of oxygen [3] by lipoxygenases (LOX) to linoleic acid hydroperoxides (LOOH **2**) (Scheme 1). Peroxylradicals (LOO*) produced as intermediates in this process resp. hydroperoxides in presence of Fe^{3+} are able to epoxidize double bonds under physiological conditions [4]. In rat liver both epoxidation of cholesterol by microsomal lipid hydroperoxides as well as their biotransformation to the corresponding 5,6-glycols was observed [5]. Biotransformation of cholesterol to 5,6-chlorohydrins was detected to occur in presence of hydrogen peroxide and chloride ions by catalysis of the haem enzyme myeloperoxidase [6] (Scheme 1). The content of epoxides in plants increases with ageing: in the essential oil of lemon balm plants (*Melissa officinalis*



Scheme 1. Production of linoleic acid hydroperoxides and generation of sterol oxidation products as result of oxidative stress and lipid peroxidation in biological tissue.

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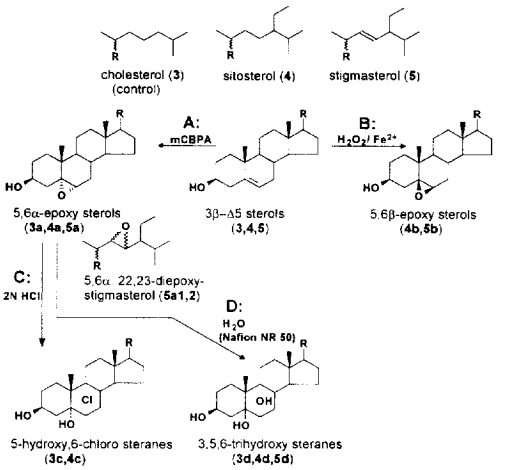
L.) an increase of the amount of caryophyllene oxide during ageing was observed combined with a simultaneously increase in lipid peroxidation [7]. In photoautotrophic suspension cell cultures of *Chenopodium rubrum* L. phytosterol epoxides and derived products were detected in higher amounts than in young cell cultures [8].

Some epoxides are allelochemicals, antifeedants or insect hormones [9, 10] and develop antifungal activity [11, 12]. The physiological properties of oxidation products of cholesterol (**3**) were studied in detail [13], they were found to be involved in numerous pathological processes e.g. carcinogenesis [14, 15] and atherogenesis [16]. Due to the reported toxicity of cholesterol epoxides we suspected that phytosterol epoxides may perform similar harmful effects. In this paper we describe the testing of oxidation products of phytosterols for general cytotoxity. The test results were checked for reliability by comparison with those of cholesterol derivatives known to develop cytotoxic properties.



Scheme 2. Synthetic pathways to oxidized phytosterols (**A**: 5,6α-epoxysterols; **B**: 5,6β-epoxysterols; **C**: 5-hydroxy, 6-chlorosterols; **D**: 3,5,6-trihydroxysterols).

RESULTS AND DISCUSSION

Synthesis of test compounds

Since the first step in the biological generation of epoxides seems to be lipid peroxidation, we prepared 13S-hydroperoxy-9,11-octadecadienoic acid (13-LOOH, **2b**) in order to check it for cytotoxity. 13-LOOH (**2b**) was synthesized by action of soy bean lipoxygenase (LOX) on linoleic acid (**1**) [17]. 13-LOOH (**2b**) was purified by preparative silica gel TLC. LOOH-activity was determined by HPLC-chemoluminescence to be 68% [18].

In order to check the reliability of the test system the cytotoxicity of phytosterol oxidation products should be compared with those of cholesterol oxidation products. Therefore, cholesterol (**3**), its 5,6α-epoxide (**3a**), 5,6-chlorohydrin (**3c**) and 3,5,6-trihydroxycholestane (**3d**) were examined for toxic properties. The phytosterols sitosterol (**4**) and stigmasterol (**5**) were tested as well as their 5,6α- (**4a**, **5a**), 5,6β-epoxides (**4b**, **5b**) and the corresponding 3,5,6-trihydroxysteranes (**4d**, **5d**).

5,6α-epoxysterols were synthesized by reaction of corresponding sterols with *m*-chloroperbenzoic acid (mCPBA) [19] (Scheme 2, **A**). The α/β-ratio of 5,6α- and 5,6β-epoxysterols (ΔRI = 10–25) was determined by GC peak-area integration and by integration of the proton resonances of H-3 and H-6 in ^{1}H NMR (5,6α-epoxide: 93–82%). In case of stigmasterol additional purification of 5,6α-epoxy-stigmasterol (**5a**) by preparative silica gel HPLC was required in order to separate the isomeric synthetic byproducts 5,6α,22,23-diepoxy-stigmasterol (**5a1,2**, 22S,23S: 22R,23R, 1:1). 5,6β-Epoxysterols were obtained by reaction of sterols with hydrogen peroxide in presence of Fe^{2+} [20] (Scheme 2, **B**). The corresponding 5,6α-epoxide:5,6β-epoxide ratio was determined to be 16–35%:84–65%.

3,5,6-trihydroxysteranes were obtained by reaction of the corresponding epoxides with water in presence of the catalyst Nafion NR 50-(H^+) [21] (Scheme 3, **D**). Reaction of the 5,6α-epoxides with 2 N HCl provided the 5-hydroxy-, 6-chloro-sterols [22] (Scheme 2, **C**).

All sterol oxidation products were purified by preparative silica gel TLC. Compounds of 90–95% purity were obtained. Identification and characterization was achieved by NMR spectroscopy and after derivatization with MSTFA by GC mass spectral analysis. The thermal instability of the chlorohydrins (**4c**,**4c**) excluded characterization by GC mass spectrometry. Therefore, their structures were secured by NMR spectroscopy and direct inlet-EI mass spectrometry. Total ^{1}H and ^{13}C signal assignment of the synthesized phytosterols was done by combination of 1D and 2D NMR techniques (^{1}HNMR, COSY, TOCSY, HMQC, HMBC, ^{13}CNMR, APT). NMR and mass spectral data of 5,6α,22,23-di-epoxy-stigmasterol (**5a1,2**) are described for the first time (^{1}H NMR data: Table 1, ^{13}C NMR data: Table 2).

The Tenebrio molitor *bioassay*

Meyer *et al.* [23] developed a bioassay for testing the cytotoxicity of plant compounds: in the so-called Brine shrimp test, the compounds were added to the brine medium of the shrimp (*Artemia salina* Leach) and the LC_{50}-value was determined. Since it is difficult to test substances which are rarely soluble in brine (e.g. lipids), we investigated the applicability of a test developed by Krieg [24] who investigated cytotoxicity by direct injection of diluted samples into the test animals. In addition, metabolism of the active compounds during resorption by the intestine could be excluded. We modified this test to investigate the toxicity of polyhydroxylated and epoxidized sterols. The biotest was carried out with meal-worms (*Tenebrio*

Table 1. ^{1}H NMR (500 Mhz) data of phytosterol oxidation products of sitosterol (S) and stigmasterol (ST): α-S-EP: 5,6α-epoxysitosterol, characteristic resonances of the β-isomer are quoted as (β), $CDCl_3$; S-HCl: 5-hydroxy, 6-chloro-sitosterol, $CDCl_3$; S-Triol: 3,5,6-trihydroxy-sitostane, CD_3OD; α-ST-EP: 5,6α-epoxystigmasterol, characteristic resonances of the β-isomer are quoted as (β), $CDCl_3$; α-ST-DiEP: 5,6α, 22,23-diepoxystigmasterol, $CDCl_3$; chemical shifts are quoted in δ units relative to TMS

H	α-S-EP	S-HCl	S-Triol	α-ST-EP	α-ST-DiEP
1a	1.36, *dd*, 1H	1.56, *dd*, 1H	1.66, *dd*, 1H	1.35, *m*, 1H	1.34, *m*, 2H
e	1.66, *dd*, 1H	1.41, *dd*, 1H	1.31, *dd*, 1H	1.66, *m*, 1H	1.65, *m*, 2H
2a	1.58, *m*, 1H	1.51, *m*, 1H	1.57, *m*, 1H	1.57, *m*, 1H	1.58, *m*, 2H
e	1.88, *m*, 1H	1.84, *m*, 1H	1.82, *m*, 1H	1.89, *m*, 1H	1.88, *m*, 2H
3a (α)	3.88, *m*, 1H (α)	4.14, *m*, 1H	4.15, *m*, 1H	3.88, *m*, 1H (α)	3.89, *m*, 2H (α)
(β)	3.67, *m*, 1H (β)			3.67, *m*, 1H (β)	3.65, *m*, 2H (β)
4a	2.05, *dd*, 1H	2.24, *dd*, 1H	2.20, *dd*, 1H	2.05, *dd*, 1H	2.05, *dd*, 2H
e	1.27, *m*, 1H	1.65, *m*, 1H	1.69, *m*, 1H	1.25, *m*, 1H	1.27, *m*, 2H
6a (α)	2.87, *d*, 1H (α)	3.83, *m*, 1H	3.57, *m*, 1H	2.87, *d*, 1H (α)	2.88, *d*, 2H (α)
(β)	3.03, *d*, 1H (β)			3.03, *d*, 1H (β)	3.03, *d*, 2H (β)
7a	1.46, *dd*, 1H	1.82, *dd*, 1H	1.54, *dd*, 1H	1.44, *dd*, 1H	1.49, *m*, 2H
e	1.89, *m*, 1H	1.95, *m*, 1H	1.76, *m*, 1H	1.885, *m*, 1H	1.87, *m*, 2H
8a	1.35, *m*, 1H	1.81, *m*, 1H	1.75, *m*, 1H	1.35, *m*, 1H	1.37, *m*, 2H
9a	1.23, *m*, 1H	1.23, *m*, 1H	1.43, *m*, 1H	1.24, *m*, 1H	1.25, *m*, 2H
11	1.36, *m*, 1H	1.33, *m*, 1H	1.27, *m*, 1H	1.36, *m*, 1H	1.35, *m*, 2H
	1.25, *m*, 1H	1.38, *m*, 1H	1.32, *m*, 1H	1.22, *m*, 1H	1.23, *m*, 2H
12a	1.11, *m*, 1H	1.16, *m*, 1H	1.08, *m*, 1H	1.11, *m*, 1H	1.12, *m*, 2H
e	1.93, *m*, 1H	2.00, *m*, 1H	1.93, *m*, 1H	1.91, *m*, 1H	1.90, *m*, 2H
14a	0.96, *m*, 1H	1.11, *m*, 1H	1.04, *m*, 1H	0.95, *m*, 1H	0.96, *m*, 2H
15a	1.54, *m*, 1H	1.54, *m*, 1H	1.52, *m*, 1H	1.52, *m*, 1H	1.0, *m*, 2H
e	0.97, *m*, 1H	1.09, *m*, 1H	1.02, *m*, 1H	0.95, *m*, 1H	1.60, *m*, 2H
16a	1.79, *m*, 1H	1.83, *m*, 1H	1.17, *m*, 1H	1.66, *m*, 1H	1.90/1.88, 2H
e	1.22, *m*, 1H	1.26, *m*, 1H	1.77, *m*, 1H	1.21, *m*, 1H	1.56/1.30, 2H
17a	1.05, *m*, 1H	1.08, *m*, 1H	1.06, *m*, 1H	1.10, *m*, 1H	1.27/1.25, 2H
18 (α)	0.58, *s*, 3H (α)	0.69, *s*, 3H	0.64, *s*, 3H	0.60, *s*, 3H (α)	0.58/0.59, 6H
(β)	0.62, *s*, 3H (β)			0.63, *s*, 3H (β)	
19 (α)	1.03, *s*, 3H (α)	1.26, *s*, 3H	1.19, *s*, 3H	1.04, *s*, 3H	1.035, *s*, 6H
(β)	0.97, *s*, 3H (β)				
20	1.33, *m*, 1H	1.34, *m*, 1H	1.29, *m*, 1H	1.98, *m*, 1H	1.25, *m*, 2H
21	0.87, *d*, 3H	0.90, *d*, 3H	0.89, *d*, 3H	0.97, *d*, 3H	0.98/0.96, 6H
22	0.98, *m*, 1H	1.31, *m*, 1H	1.31, *m*, 1H	5.11, *dd*, 1H	2.45/2.48, 2H
	1.30, *m*, 1H	1.00, *m*, 1H	0.98, *m*, 1H	—	—
23	1.63, *m*, 2H	1.15, *m*, 2H	1.20, *m*, 1H	4.98, *dd*, 1H	2.47/2.70, 2H
			1.25, *m*, 1H	—	—
24	0.92, *m*, 1H	0.91, *m*, 1H	0.88, *m*, 1H	1.50, *m*, 1H	0.83/0.97, 2H
25	1.65, *m*, 1H	1.23, *m*, 1H	1.15, *m*, 1H	1.50, *m*, 1H	1.82, *m*, 2H
26	0.81, *d*, 3H	0.80, *d*, 3H	0.79, *d*, 3H	0.73, *d*, 3H	0.90, *d*, 6H
27	0.79, *d*, 3H	0.82, *d*, 3H	0.81, *d*, 3H	0.81, *d*, 3H	0.92, *d*, 6H
28	1.21, *m*, 2H	1.65, *m*, 2H	1.61, *m*, 2H	0.81, *m*, 1H	0.8, *m*, 2H
				1.16, *m*, 1H	1.32, *m*, 2H
29	0.82, *t*, 3H	0.83, *t*, 3H	0.82, *t*, 3H	0.77, *t*, 3H	0.94, *t*, 6H

molitor L.). For each biotest 20 animals were chosen. After injection of 2 μl test solution into each individual the mortality was determined after 1, 2, 3, 4, 5 and 23 hr. Preliminary tests showed that injury with the injection needle of the 5 μl Hamilton syringe did not reduce the vitality of the test animals: all reference animals survived during the test period of 23 hr. Next, the solvent systems were tested in order to determine

Table 2. ^{13}C NMR (125.76 Mhz) data of the synthesized sterol oxidation products: α-S-EP: 5,6α-epoxysitosterol, $CDCl_3$; S-HCl: 5-hydroxy,6-chloro-sitosterol, $CDCl_3$; S-Triol: 3,5,6-trihydroxysitostane, CD_3OD; α-ST-EP: 5,6α-epoxystigmasterol, $CDCl_3$; α-ST-DiEP: 5,6α,22,23-diepoxystigmasterol, $CDCl_3$; chemical shifts are quoted in δ units relative to TMS.

C	α-S-EP	S-HCl	S-Triol	α-ST-EP	αST-DiEP
1	32.39	32.93	33.51	32.39	32.38/32.40
2	31.09	30.68	31.87	31.07	31.08/31.08
3	68.73	67.66	68.26	68.70	68.70/68.71
4	39.86	41.85	41.71	39.85	39.83/39.86
5	65.66	76.91	76.59	65.68	65.64/65.65
6	59.28	63.82	76.47	59.27	59.18/59.24
7	28.81	35.49	35.43	28.79	28.75/28.77
8	29.88	30.10	31.58	29.87	29.88/29.89
9	42.55	45.74	46.44	42.57	42.64/42.67
10	34.84	39.02	39.31	34.85	34.84/34.85
11	20.63	21.14	22.26	20.62	20.59/20.64
12	39.39	39.82	41.36	39.28	39.19/39.30
13	42.32	42.70	43.77	42.20	42.55/42.63
14	56.84	56.07	57.36	56.94	56.43/56.46
15	24.04	24.10	25.18	24.10	24.25/24.29
16	28.08	28.19	29.33	28.73	26.87/27.77
17	55.77	55.38	57.31	55.61	53.12/55.76
18	11.85	12.13	12.66	12.03	11.84/11.98
19	15.35	18.40	17.41	15.91	15.90/15.90
20	26.12	36.14	37.32	40.45	38.70/38.84
21	18.68	18.69	19.35	21.15	16.17/16.27
22	33.88	33.87	34.94	138.21	62.17/63.07
23	26.08	26.02	24.03	129.30	58.55/62.05
24	45.81	45.77	47.03	51.19	48.27/48.76
25	29.12	23.01	26.99	31.85	29.13/29.31
26	19.01	19.00	20.28	18.96	19.34/19.41
27	19.81	19.81	19.47	21.07	19.51/20.19
28	23.04	29.07	30.20	25.38	20.82/20.91
29	11.96	11.95	12.41	12.23	12.36/12.46

their influence on the survival of the animals. Injections of 2 μl of pure ethanol, DMSO, *N*,*N*-dimethylformamide, non-sterilized H_2O, sterilized H_2O, H_2O–1% Tween 80, H_2O–1% Tween 20 were tested.

Organic solvents caused high mortalities and ranged from 35% (*N*,*N*-dimethylformamide) 60% (DMSO) up to 100% (ethanol). Non-sterilized water effected a 20% mortality, too. Sterile water showed no mortality, but it turned out to be an unsuitable solvent because the test substances are lipophilic. Therefore, an emulsifier system containing H_2O (sterile)–Tween 80 (1%), was tested, which showed good solubility but caused 30% mortality. In contrast H_2O (sterile)–Tween 20 (1%) provided enough solubility without mortality.

Therefore, 1×10^{-2}, 5×10^{-3}, 1×10^{-3} up to 1×10^{-5} M emulsions of the test substances in H_2O–1% Tween 20 were prepared. Each test was carried out with 20 meal-worms. Two microlitres of the emulsions were injected into each meal-worm. It was assumed that the concentration of the test substance in the meal-worm was in a physiological range after distribution as shown for hydroperoxides in blood plasma [18]. Control tests were performed with pure solvent (1% Tween 20 in H_2O). The mortality was measured after 1, 2, 3, 4, 5 and 23 hr by counting the dead individuals.

At first 13*S*-linoleic acid hydroperoxide (13-LOOH, **2b**) was tested in concentrations from 1×10^{-5} up to 1×10^{-2} M. 13S-LOOH **2b** showed distinct cytotoxic activity. Furthermore, a relation between mortality and substance concentration was observed (Fig. 2).

Lipid hydroperoxides are involved in numerous pathological processes, in particular during inflam-

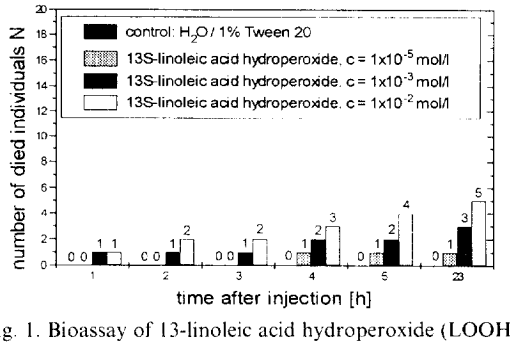


Fig. 1. Bioassay of 13-linoleic acid hydroperoxide (LOOH): number of dead individuals (*Tenebrio molitor* L.) in relation to time after injection measured for different concentrations. Number of test individuals: $N = 20$.

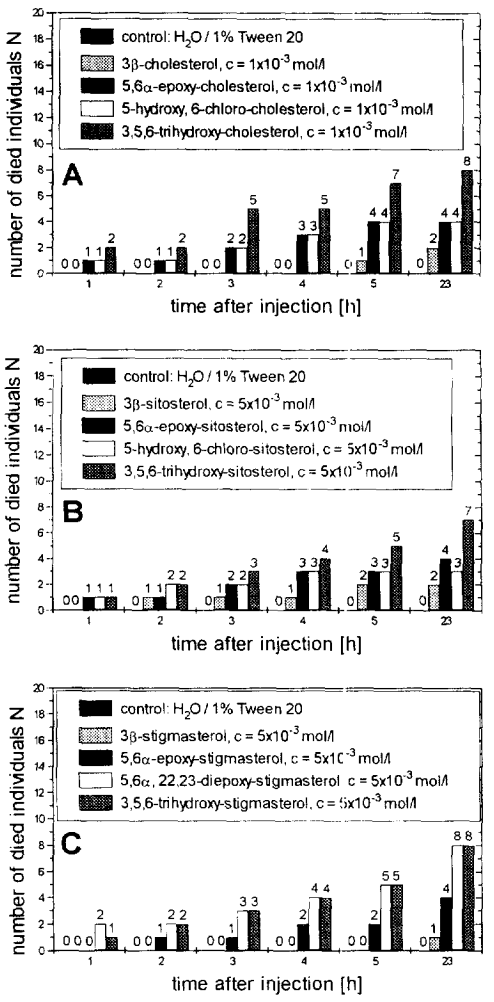


Fig. 2. Control of the reliability of the *Tenebrio molitor* assay by testing cholesterol oxidation products ($c = 1 \times 10^{-3}$ M) with known cytotoxicity (A); *Tenebrio molitor* assay of phytosterols and corresponding oxidation products presented for sitosterol ($c = 5 \times 10^{-3}$ M, B) and sigmasterol ($c = 5 \times 10^{-3}$ M, C) derivatives.

mation and chronic disease [25]. Furthermore, an increase of lipid hydroperoxides can be detected in ageing [2]. Besides this they are found in rotten food, e.g. the toxic properties of rancid fats are assigned to lipid hydroperoxides [26].

The reliability of the test system was checked by comparing the *Tenebrio molitor* test results of phytosterols with those of cholesterol derivatives. Cholesterol epoxides and also their hydrolysis products, 3,5,6-trihydroxycholestanes, are cytotoxic [27]. Furthermore, cholesterol epoxides bind to DNA [28] and react with SH-nucleophiles, e.g. glutathione [29]. Further cholesterol oxidation products of biological interest are the 5,6-chlorohydrins which are also cytotoxins [6]. Cholesterol (**3**) showed only at the end of the test period (23 hr) low toxicity (10%). In case of 5,6α-epoxycholesterol (**3a**) cytotoxic activity was observed already after 1 hr, at hour 23 the mortality rate had raised to 20%. The same result was obtained for 5-hydroxy,6-chlorocholesterol (**3c**). In accordance to the known activity of 3,5,6-trihydroxycholestane (**3d**) the mortality rates reached the highest values (40%, 23 hr) of all tested substances [Fig. 2(A)]. These findings proved that the *Tenebrio molitor* assay is a suitable test system for cytotoxity.

Testing of the corresponding sitosterol derivatives **4**, **4(a–d)** provided comparable results. The overall mortality was five times lower than that of the corresponding cholesterol derivatives. 3,5,6-Trihydroxysitostane (**4d**: 5×10^{-3} M, 23 hr, 35% mortality) turned out to be the most active of the oxidized sitosterol derivatives. 5,6α-Epoxy- (**4a**) and 5-hydroxy,6-chloro-sitosterol (**4c**: 5×10^{-3} M, 23 hr, 15% mortality) had both half of the activity of 3,5,6-trihydroxysitostane (**4d**) [Figure 2(B)].

Analogous results were obtained from the stigmasterol derivatives: stigmasterol (**5**), 5,6α-epoxystigmasterol (**5a**), 5,6β-epoxystigmasterol (**5b**) and 3,5,6-trihydroxy-stigmastane (**5d**). The activities of the stigmasterol derivatives ranked in the same order as corresponding sitosterol derivatives [Fig. 2(C)]. Additional testing of the synthesized 5,6α, 22, 23-diepoxystigmasterol (**5a1,2**) revealed a mortality of 40% which is twice the activity compared to the 5,6α-monoepoxide (**5a**: 5×10^{-3} M, 23 hr, 20%). It showed the same mortality as 3,5,6-trihydroxystigmastane [**5d**: 5×10^{-3} M, 23 hr, 40%, Fig. 2(C)].

On comparing the toxicity of 5,6α-epoxides and 5,6β-epoxides of sitosterol (**4a**, **4b**) and stigmasterol (**5a**, **5b**) no significant difference in the mortality on *Tenebrio molitor* larvae was detected. Both isomers showed almost the same activity.

The epoxidation process of sterols was studied in more detail after detection of cholesterol oxidation products in atherosclerotic plaques [27]. Furthermore, involvement of cholesterol oxidation products in carcinogenesis [14, 15] was reported. Cholesterol epoxides (**3a**, **3b**) are produced by incubation of cholesterol with linoleic acid–lipoxygenase [30] or by reaction with linoleic acid hydroperoxides in presence of Fe^{2+}-ions [31]. Epoxidation *in vivo* is promoted by action of enzymes, e.g. epoxidases or cytochrome P-450 [32]. Special α-epoxidases produce 5,6α-epoxycholesterol [33]. In rat liver cholesterol 5,6β-epoxide is generated by microsomal lipid hydroperoxides [31]. The epoxide is biotransformed to the corresponding 5,6-glycol [34]. In addition epoxide hydrolase activity was reported from mammalian tissue [35, 36]. Metabolic activation and detoxification of epoxides *in vivo* is also achieved by the enzyme glutathione *S*-transferase (GST) [29] which catalyses the formation of glutathione conjugates. These conjugates are catabolized to mercapturic acid metabolites which were detected in urine [37]. In case of incomplete detoxification epoxides react with cell components like membranes [38], DNA [28], RNA [39] and peptides [40]. Similar reactions might occur in plants.

Increases of oxidized sterols were detected after storage of food [41, 42] or after drying of plant

material [43]. Oxidized sitosterol derivatives were isolated from soybean oil and from wheat flour [44]. Oxidation of stigmasterol was observed in heated triacylglycerols [45]. Chlorohydrins of sitosterol glycosides were isolated from the cockroach *Blattella germanica* L. [46].

In contrast to the known cytotoxity of oxidized cholesterol derivatives [6, 27] little is known about the biological activities of oxidized phytosterols. It is remarkable that the oxidized phytosterols showed comparable cytotoxic effects in the *Tenebrio molitor* tests, but their activity was 5-fold lower compared to the corresponding cholesterol derivatives. Cholesterol **3** and the phytosterols sitosterol **4** and stigmasterol **5** differ only in the side-chain, which is of obvious biological importance. Phytosterol-enriched diets could decrease the effects of artheriosclerosis and inflammation [47]. 3,5,6-cholestantriol **3d** is an inhibitor of cell proliferation [48]. In aged cell cultures (stationary phase) an increase of the amount of phytosterol 3,5,6-triols was detected [8]. Perhaps they perform analogous effects.

On the other hand anti-juvenile hormones (precocenes) from plants are epoxidized in the corpora allata of insects. These epoxides react with cellular proteins. This causes the cell death of the affected tissue [49]. Epoxides from unsaturated fatty acids were reported to develop high antifungal activity [11, 12]. In regard of these observations and the results reported here on the action of sterol epoxides on meal worms, we suspect that epoxides of phytosterols may also be able to serve as toxins in plant–fungal or plant–insect interactions.

EXPERIMENTAL

Materials. *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) was obtained from Macherey and Nagel (Düren). Sterols were purchased from Aldrich (cholesterol, **3**), Merck (sitosterol, **4**) and Fluka (stigmasterol, **5**). Solvents, obtained from Merck (Darmstadt), were distilled before use. TLC was performed on home made 0.75 mm PF_{254} silica gel 60 (Merck, Darmstadt) plates. Silica gel-HPLC was performed on Beckman System Gold apparature: (Programmable Solvent Module 125, Diode Array Detector Module 168, Control unit: PC-software: The Personal Chromatograph V601, Reodyne injection valve 7125; sample loop: 500 μl; prep. column: 250 × 20 mm, Ultrasep FS 100, 6 μm (Bischoff Chromatography, Leonberg); solvents: *n*-hexane; 2-propanol). NMR-experiments were performed with chloroform-d_1, acetone-d_6, and methanol-d_4 as solvents.

GC-, GC-MS and *DIEIMS analysis*: Gas–liquid chromatography (GC) was carried out an a United Technologies Packard Model 438S chromatograph equipped with a flame ionization 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 and W Scientific, Mainz-Kastel); carrier gas: H_2; splitting ratio: 1:10; injector temp. 270°; detector temp. 290°; temp. programme: 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 mixt. of *n*-alkanes (C_{10}–C_{40}) [50].

Compound identification was achieved by GC-MS: Gas chromatograph HP 5890 series II, fused-silica DB-1 capillary column (30 m × 0.32 mm i.d., film thickness 0.1 μm (J and 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); ionization energy: 70 eV. Direct inlet-electron impact-MS (DIEMS) was performed on a Finnigan MAT 8500 mass spectrometer, data system MAT SS 200, ionization energy: 70 eV.

Enzymatic synthesis of 13-linoleic acid hydroperoxide (13-LOOH, **2b**). Achieved according to the procedure of Teng and Smith [17]. Before use the product was purified by prep. silica gel TLC (solvent system: *n*-hexane–Et_2O–HOAc, 3:1:0.1). The LOOH area (R_f = 0.25–0.35)—containing the hydroperoxide—was eluted with EtOAc [yield 1.25 g (4.00 mmol) 13-LOOH, 61.5%]. The purified LOOH was dissolved in 100 ml MeOH and stored under Argon at −18°. 5 mg LOOH were reduced with $NaBH_4$, esterified with diazomethane, trimethylsilylated with MSTFA and subjected to GC-MS analysis to check purity (87% hydroxyoctadecadienoic acid (9-HODE, 13-HODE), ratio of isomers: 89:11 (13-HODE:9-HODE). Activity of non-reduced LOOH was determined to be 68.19% by means of HPLC-chemoluminescence [18] (RP18-HPLC: column: 250 × 4 mm, Spherisorb ODS II, 5 μm (Bischoff Chromatogr., Leonberg) solvents: A: H_2O–0.1% HOAc, B: MeOH–0.1% AcOH; HPLC-programme: 70% B (8 min, isocratic), gradient to 83% B (3 min), 83% B (isocratic), LOOH R_t = 15.5 min (T = 25°), detection: post column reaction with luminol–cytochrome C, Soma Chemi Lumi Detector S-3400 (300–650 nm).

Synthesis of 5,6α-epoxysterols. (Kametani *et al.* [19], modified, as described in [8]): In case of epoxidation of stigmasterol an additional purification step by prep. silica gel HPLC was required (column: 250 × 20 mm, Ultrasep FS 100, 6 μm (Bischoff Chromatography, Leonberg); Flow 10.0 ml min^{-1}, *n*-hexane–2-propanol 10:1, isocratic, UV-detection: 212 nm), in order to separate simultaneously generated 5,6α,22,23-diepoxy-stigmasterol **5a1,2**. The purified products were examined by means of NMR spectroscopy (^1H, ^{13}C, COSY, TOCSY, HMQC, HMBC) and subjected to GC-MS analysis after trimethylsilylation.

5,6α-epoxycholesterol (**3a**). (yield: 0.76 g, 76%), TLC (Polygram SIL G/UV 254, cyclohexane:EtOAc, 1:1): R_f = 0.47, TMS-derivative: GC (DB-1): R_I = 3209; GC-EIMS, ^1H NMR and ^{13}C NMR data are in agreement with literature data [8, 33, 51, 52). *5,6α-epoxysitosterol* (**4a**). Yield: 0.94 g, 94%. TLC

(Polygram SIL G/UV 254, cyclohexane:EtOAc, 1:1, $R_f = 0.41$, TMS-derivative: GC(DB1): $R_I = 3431$; GC-EIMS 70 eV, *m/z* (rel. int.) 502[M]$^+$ (54), 487[M–Me]$^+$ (25), 484[M–H_2O]$^+$ (22), 412[M–TMSOH]$^+$ (69), 394[M–TMSOH–H_2O] (100), 379[M–TMSOH–H_2O–Me]$^+$ (25), 253[M–TMSOH–H_2O–141] (28); ^1H and ^{13}C NMR data see Tables 1 and 2. *5,6α-epoxystigmasterol* (**5a**). Yield: 0.43 g, 43%. TLC (Polygram SIL G/UV 254, cyclohexane:EtOAc, 1:1): $R_f = 0.48$, TMS-derivative: GC(DB1): $R_I = 3361$; GC-EIMS 70 eV, *m/z* (rel. int.) 500[M]$^+$ (50), 485[M–Me]$^+$ (16), 482[M–H_2O] (24), 410[M–TMSOH]$^+$ (51), 392[M–TMSOH–H_2O]$^+$ (54), 377[M–TMSOH–H_2O–Me]$^+$ (12), 253[M–TMSOH–H_2O–139]$^+$ (100); ^1H and ^{13}C NMR data see Tables 1 and 2. *5,6α. 22,23,-diepoxystigmasterol* (**5a1,2**). Yield: 0.21 g, 21%. TLC (Polygram SIL G/UV 254, cyclohexane:EtOAc, 1:1): $R_f = 0.45$, GC(DB1): TMS-derivative: GC(DB1): $R_I = 3532$, 3551; GC-EIMS 70 eV, *m/z* (rel. int) 516[M]$^+$(87), 501[M–Me]$^+$ (18), 498[M–H_2O]$^+$ (28), 431[M–85]$^-$ (13), 426[M–TMSOH]$^+$ (57), 408[M–TMSOH–H_2O]$^+$ (36), 341[M–TMSOH–85]$^+$ (20), 253[M–TMSOH–H_2O–155] (41), 127 (100); ^1H and ^{13}C NMR data see Tables 1 and 2.

Synthesis of 5,6β-epoxysterols by reaction with Fe (acac)$_3$–H_2O_2 [20]. In a 250 ml flask fitted with a sepn funnel and a condenser 1.87 g (5.3 mmol) ferric acetylacetonate (Fluka) and 0.5 mmol sterol were dissolved in 150 ml MeCN. Then 15 ml H_2O_2 (perhydrol, Merck, 30%) were added dropwise. After complete reaction (TLC control) the excess of oxidant was destroyed by addition of a satd aq. Na_2SO_3 soln followed by extraction with EtOAc. The organic phase was washed with satd NaCl soln and dried over anhydrous Na_2SO_4. After removal of solvent the residue was purified by prep. silica gel TLC (cyclohexane: EtOAc, 1:1).

5,6β-epoxysitosterol (**4b**). Yield: 0.90 g, 48%, TLC (Polygram SIL G/UV 254, cyclohexane:EtOAc, 1:1): $R_f = 0.47$, TMS-derivative: GC(DB1): $R_I = 3420$; GC-EIMS 70 eV, *m/z* (rel. int.) see *5,6α-epoxysitosterol* (**4a**); ^1H NMR data see Table 1. *5,6β-epoxystigmasterol* (**5b**). Yield: 0.57 g, 28%. TLC (Polygram SIL G/UV 254, cyclohexane:EtOAc, 1:1): $R_f = 0.48$, TMS-derivative: GC(DB1): $R_I = 3340$; GC-EIMS 70 eV, *m/z* (rel. int.) see *5,6α-epoxystigmasterol* (**5a**).

Synthesis of 5-hydroxy,6-chloro sterols by hydrolysis of 5,6-epoxy sterols with 2 N HCl [22, modified]. 100 mg, 5,6α-epoxysterol were dissolved in 40 ml Et_2O. 20 ml 2 N HCl were added. The soln was stirred 24 hr at 20°. After extraction with Et_2O the organic phase was dried over anhydrous Na_2SO_4. The solvent was removed and the residue was purified by prep. silica gel TLC (cyclohexane:EtOAc, 1:1; $R_f = 0.35$–0.45). The thermical instability of the chlorohydrins excluded GC-MS analysis (injector temp.: 290°). Characterization was achieved by direct inlet electron impact-MS (DI-EIMS) and NMR spectroscopy. *5-hydroxy,6-chloro-cholesterol* (**3c**). Yield: 0.68 g, 68%. TLC (Polygram SIL G/UV 254, cyclohexane:EtOAc, 1:1): $R_f = 0.41$, DI-EIMS 70 eV, *m/z* (rel. int.) 440[M+2(^{37}Cl)]$^+$ (8), 438[M(^{35}Cl)]$^+$ (15), 420[M–H_2O]$^+$ (5), 402[M–HCl]$^+$ (12), 384[M–H_2O–HCl]$^+$ (100), 369[384–Me] (21), 366[384–H_2O] (19), ^1H and ^{13}C NMR data see Tables 1 and 2. *5-hydroxy,6-chlorositosterol* (**4c**). Yield: 0.72 g, 72%. TLC (Polygram SIL G/UV 254, cyclohexane:EtOAc, 1:1): $R_f = 0.38$, DI-EIMS 70 eV, *m/z* (rel. int.) 468[M+2(^{37}Cl)]$^+$ (3), 466[M(^{35}Cl)]$^+$ (8), 448[M$^+$–H_2O]$^+$ (30), 430[M–HCl]$^+$ (80), 412[M–H_2O–HCl]$^+$ (100), 397[412–Me] (32), 394[412–H_2O] (27), ^1H and ^{13}C NMR data see Tables 1 and 2.

Synthesis of 3β,5α,6β-trihydroxy-sterols. (according to [21], modified as described in [8]): *3β,5α,6β-trihydroxycholesterol*: (**3d**) (yield: 0.90 g, 45%), TLC (Polygram SIL G/UV 254, cyclohexane:EtOAc, 1:4): $R_f = 0.19$; TMS-derivative: GC (DB-1): $R_I = 3405$, GC-EIMS, ^1H and ^{13}C NMR data are in agreement with literature data [8, 51, 52]. *3β,5α,6β-trihydroxysitosterol* (**4d**). Yield: 0.72 g, 36%. TLC (Polygramme SIL G/UV 254, cyclohexane:EtOAc, 1:4): $R_f = 0.21$; TMS-derivative: GC (DB-1): $R_I = 3520$; GC-EIMS 70 eV, *m/z* (rel. int.) 664[M]$^+$ (0), 574[M–TMSOH]$^+$ (68), 559[M–TMSOH–Me]$^+$ (37), 484[M–2x TMSOH]$^+$ (100), 469[M–2x TMSOH–Me]$^-$ (39), 431 (73), 394[M–3x TMSOH]$^+$ (40), 379[M–3x TMSOH–Me)$^+$ (12), 349 (46), 253[M–3x TMSOH–141]$^+$ (17). *3β,5α,6β-trihydroxy-stigmasterol* (**5d**). Yield: 0.63 g, 32%. TLC (Polygramme SIL G/UV 254, cyclohexane:EtOAc, 1:4): $R_f = 0.17$; TMS-derivative: GC (DB-1): $R_I = 3446$; GC-EIMS 70 eV, *m/z* (rel. int.) 662[M]$^+$ (0), 572[M–TMSOH]$^+$ (82), 557[M–TMSOH–Me]$^+$ (30), 482[M–2x TMSOH]$^-$ (100), 467[M–2x TMSOH–Me]$^+$ (34), 429 (73), 392[M–3x TMSOH]$^+$ (28), 377[M–3x TMSOH–Me]$^+$ (6), 347 (20), 253[M–3x TMSOH–139]$^+$ (59).

Bioassay for cytotoxity of oxidized phytosterols. (developed according to Krieg [24]). Test compounds—diluted in H_2O–Tween 20 (1%)—were applied to meal-worms (larvae of *Tenebrio molitor* L.) by injection with a Hamilton 5 μl syringe. Mortality after injection was measured. As a result of testing of pure solvents (EtOH, DMSO, *N,N*-dimethylformamide, H_2O (non-sterile), H_2O (sterile), H_2O–1% Tween 80, H_2O–1% Tween 20) the H_2O–1% Tween 20 system was selected to be best suited for the testing of 13-linoleic acid hydroperoxide (13-LOOH, **2b**), phytosterols (cholesterol **3**, sitosterol **4**, stigmasterol **5**), 5,6α-epoxysterols (5,6α-epoxycholesterol **3a**, 5,6α-epoxysitosterol **4a**, 5,6-epoxystigmasterol **5a**; 5,6α,22,23-diepoxystigmasterol **5a1,2**), 5,6β-epoxysterols (5,6β-epoxysitosterol **4b**, 5,6β-epoxy-stigmasterol **5b**), chlorohydrins (5-hydroxy-,6-chlorocholesterol **3c**, 5-hydroxy-,6-chloro-sitosterol **4c**), 3,5,6-trihydroxy-steranes (3,5,6-trihydroxycholestane **3d**, 3,5,6-trihydroxy-sitostane **4d**, 3,5,6-trihydroxystigmastane **5d**). 20 meal-worms were used per test. 2 μl of 1×10^{-2} M, 5×10^{-3} M and 1×10^{-3} M emulsions in H_2O–1% Tween 20 of the test substances were injected into the meal-worms. Control tests were car-

ried out with pure solvent (1% Tween 20 in H_2O). The mortality was measured after 1, 2, 3, 4, 5 and 23 hr by counting the dead individuals. All tests were repeated $\times 3$.

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