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Oxygenated Sterol Derivatives. Their Identification from the Fungus-Infected Silkworm Carcass, *Bombyx cum Botryte*, and Their Effects on Growth and Sterol Metabolism of the Silkworm, *Bombyx mori*

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Several oxygenated sterols, *e.g.* ergosterol peroxide, 7-oxocholesterol and 7β-hydroxycholesterol, were identified from the fungus-infected carcass of silkworm, *Bombyx cum Botryte*. However, they were nontoxic to the silkworm *Bombyx mori* reared on a diet containing these oxygenated sterols (0.01%) together with sitosterol or cholesterol (0.1%).

Keywords——ergosterol peroxide; 7β-hydroxycholesterol; 15-oxo-5α-cholest-8(14)-en-3β-ol; *Bombyx cum Botryte*; *Bombyx mori*

Oxygenated sterol derivatives such as 7-oxocholesterol (**2**), 7α- and 7β-hydroxycholesterol (**3** and **4**) and 25-hydroxycholesterol (**9**) have been shown to be potent inhibitors of sterol biosynthesis and of β-hydroxy-β-methylglutaryl (HMG) coenzyme A (CoA) reductase activity when added to mammalian cultured cells.[2] Following the blockage of sterol synthesis, the concentration of sterol in the cells declines, growth ceases, and eventually the cells die. On the other hand, Cheng *et al.* isolated ergosterol peroxide (**1**), 7α-hydroxycholesterol (**3**) and 7β-hydroxy-cholesterol, -campesterol, and -sitosterol (**4**—**6**) from *Bombyx cum Botryte*, consisting of silkworms (*Bombyx mori*) killed by infection with the microscopic fungus *Botrytis bassiana* Bals.[3] The "animal drug" *B. cum Botryte* (殭蚕) has been used in China for treatment of several diseases including cancer, and some of the above-mentioned 7-hydroxylated compounds and other oxygenated sterols were reported to be cytotoxic to rat hepatoma cells.[3,4]

We considered that the death of silkworms following fungus infection might be due to the cytotoxic effect of the oxygenated sterols, which may be produced by a cooperative action of the host (silkworm) and invader (fungus). However, since the insect lacks *de novo* sterol synthesis, these compounds should be nontoxic to the insect if HMG CoA reductase is the sole target enzyme involved in the inhibitory effect of oxygenated sterols. In order to examine these possibilities, we first attempted to confirm the presence of oxygenated sterols in *B. cum Botryte*, and then the effects of several oxygenated cholesterol derivatives on the growth and sterol metabolism of the silkworm *B. mori* were investigated.

The lipid extract of *B. cum Botryte* was fractionated by column chromatography on silica gel into fractions I (sterol esters and triglycerides), II (fatty acids), III (free sterols) and IV (polar materials) in the order of elution from the column. Fraction I was saponified to give free sterols (IS) and fatty acids (IF). The samples of IS, III and IV were analyzed by gas chromatography-mass spectrometry (GC-MS) as the trimethylsilyl (TMS) ethers (Table I). As shown in Fig. 1, the major sterols in fraction III and IS were cholesterol and sitosterol. Minute amounts of campesterol and a triterpene, lupeol, were also detected. The sterol profile was essentially identical with that observed in the silkworm *B. mori*.[5] GC-MS analysis of fraction

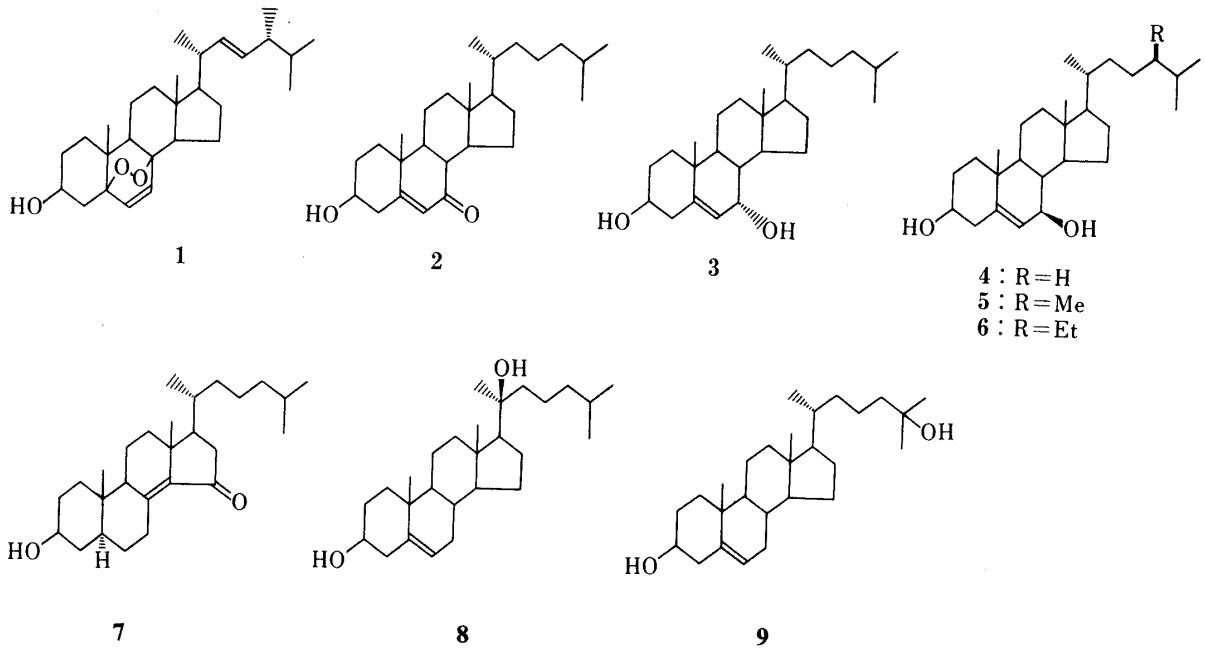

Chart 1

TABLE I. Retention Times on GC and Prominent Ions in the Mass Spectra of Sterol Trimethylsilyl Ethers

Sterol	t_R[a] (min)	m/z[b]
Cholesterol	3.7	458 (M^+), 329 (M−129), 129
Campesterol	4.5	472 (M^+), 343 (M−129), 129
Ergosterol	4.5	468 (M^+), 363 (M−90−15), 337
Stigmasterol	4.8	484 (M^+), 394 (M−90), 255
Sitosterol	5.4	486 (M^+), 357 (M−129), 129
7α-Hydroxycholesterol	3.0	546 (M^+), 456 (M−90)
7β-Hydroxycholesterol	4.0	546 (M^+), 456 (M−90)
(20*S*)-20-Hydroxycholesterol	4.6	461 (C_1–C_{21}), 201 (C_{20}–C_{27})
25-Hydroxycholesterol	6.1	546 (M^+), 131 (C_{25}–C_{27})
7-Oxocholesterol	8.0	472 (M^+), 129

a) GC was carried out on 1.5% OV-17 (1 m × 3 mm) at 275 °C.
b) Mass spectra were recorded with an LKB-9000S GC-mass spectrometer at 70 eV. The underlined m/z is base peak.

IS (Fig. 1B) indicated the presence of, (in addition to cholesterol and sitosterol) ergosterol which apparently arises from the infected fungus *Botrytis bassiana*. From fraction IV, ergosterol peroxide (**1**) was isolated in crystalline form; the mp and proton nuclear magnetic resonance (^{1}H-NMR) were in good agreement with the reported data.[6] The TMS ether of **1** seemed to be decomposed on GC as demonstrated by the finding of several peaks, of which the major peak showed the expected mass fragment ions including M^+ 500 (Fig. 2). Since no such GC-MS spectrum was obtained from any component of fraction IS, ergosterol peroxide should occur in *B. cum Botryte* as such, and not in the ester form. In contrast, ergosterol mainly existed as fatty acid esters (*vide supra*). The fatty acid compositions of fractions II and IF were analyzed as the methyl esters by GC-MS. No significant difference was observed between II and IF, and palmitic acid, stearic acid, oleic acid, linoleic acid and linolenic acid

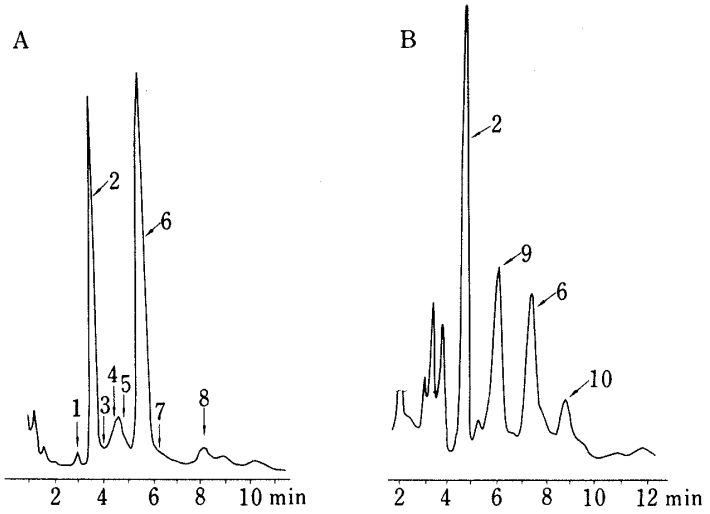

Fig. 1. GC-MS Analysis of *Bombyx cum Botryte* Sterols as Their TMS Ethers

A, sterols in fraction III (free sterol); B, sterols in fraction IS (sterol ester saponified). GC was done on a column of 1.5% OV-17 (1 m × 3 mm) at 275 °C (A), or 3% OV-17 (1 m × 3 mm) at 266 °C (B). Peak 1, 7α-hydroxycholesterol; peak 2, cholesterol; peak 3, 7β-hydroxycholesterol; peak 4, campesterol; peak 5, 7β-hydroxycampesterol, peak 6, sitosterol; peak 7, 7β-hydroxysitosterol; peak 8, 7-oxocholesterol; peak 9, ergosterol; peak 10, lupeol.

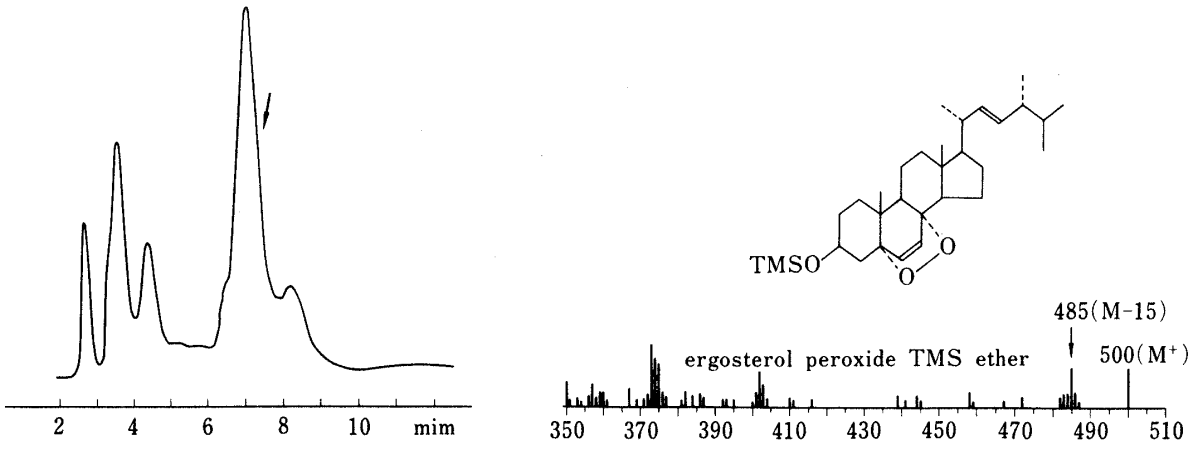

Fig. 2. GC-MS of Ergosterol Peroxide TMS Ether

GC was carried out on 3% OV-17 (1 m × 3 mm) at 253 °C.
The MS was recorded at the arrow.

were identified. The concentration of linoleic acid appeared to be higher than in the silkworm *B. mori*.[7]

In searching for other oxygenated sterols, we extensively examined the GC-MS data for fraction III (Fig. 1A), with the aid of reference compounds: 7α- and 7β-hydroxycholesterol (**3** and **4**), (20*S*)-20-hydroxycholesterol (**8**), 25-hydroxycholesterol (**9**) and 7-oxocholesterol (**2**) (see the chart and Table I). Peak 3 and peak 8 in Fig. 1A were identical with the TMS ethers of **4** and **2**, respectively. Peak 5 may be due to 7β-hydroxycampesterol TMS ether, based on the fragment at *m/z* 560 (M^+) and 470 (M-90). Similarly, peak 7 (showing *m/z* 574 and 484) may be due to 7β-hydroxysitosterol TMS ether. The retention time of peak 1 seemed to coincide

with that of 7α-hydroxycholesterol TMS ether. However, we unfortunately failed to take the MS of this peak because we had not noticed that hydroxycholesterol TMS ether runs faster than cholesterol TMS ether on GC. (20*S*)-20-Hydroxycholesterol TMS ether and 25-hydroxycholesterol TMS ether were searched for in the selected ion monitoring mode utilizing the fragment ions indicated in Table I. Although this method is much more sensitive (*ca.* thousand times) than the conventional GC-MS, these compounds were not detected in any fraction, IS, III or IV.

Because the oxygenated sterols **1**—**6** have now been identified in *B. cum Botryte, albeit* in very minute amounts (less than 1% of total sterol), we next tested these compounds for toxicity to the silkworm *B. mori*. It is well established that *B. mori* can be reared on an artificial diet supplemented with sitosterol, cholesterol or certain analogs.[8] When one of the oxygenated sterols, **1**, **2**, **4**, **7**, **8** or **9**, was added to diet as the sole sterol source, all of the larvae died within one week without molting to the second instar. Therefore, these compounds are wholly inadequate insect nutrient sterols. Then, the larvae were reared on a diet which contained 0.1% cholesterol or sitosterol in combination with 0.01% oxygenated sterol (**1**, **2**, **4**, **7**, **8**, or **9**). Most of the larvae developed to the third instar and survived at least till day 20 after hatching. Their mean body weights were not very different from the control value (Table II). 7-Oxocholesterol (**2**) even showed a growth-promoting effect when the basal sterol was sitosterol (No. 10). Some growth retardation was observed with the combination of the 15-ketone (**7**) with cholesterol (No. 5), and that of 7β-hydroxycholesterol (**4**) with sitosterol (No. 11).

In agreement with the slight growth effect of the oxygenated sterols, the sterol profiles in the larvae fed these diets were not significantly different from that of the control. Thus, the major sterols of No. 8—14 (Table II), as analyzed by high pressure liquid chromatography (HPLC) and GC-MS of the TMS ethers,[8] were always sitosterol and cholesterol; the ratio (*ca.* 1 : 1) of cholesterol to sitosterol of No. 8 (control) was essentially the same as those in No. 9—14. An example of HPLC and GC analyses is shown in Fig. 3. We can conclude that dealkylation of sitosterol in *B. mori* is not inhibited by the oxygenated sterols tested.

TABLE II. Effect of Oxygenated Sterols on the Growth of Silkworm, *Bombyx mori*

No.	Basal sterol (0.1%)	Oxygenated sterol added (0.01%)	No. of surviving larvae	Mean Bd. wt. (mg)
1	Cholesterol	None	40	59
2	Cholesterol	Ergosterol peroxide	40	60
3	Cholesterol	7-Oxocholesterol	39	58
4	Cholesterol	7β-Hydroxycholesterol	39	55
5	Cholesterol	15-Oxo-5α-cholest-8(14)-en-3β-ol	35	56
6	Cholesterol	(20*S*)-Hydroxycholesterol	40	60
7	Cholesterol	25-Hydroxycholesterol	39	60
8	Sitosterol	None	40	57
9	Sitosterol	Ergosterol peroxide	40	55
10	Sitosterol	7-Oxocholesterol	40	77
11	Sitosterol	7β-Hydroxycholesterol	38	28
12	Sitosterol	15-Oxo-5α-cholest-8(14)-en-3β-ol	36	54
13	Sitosterol	(20*S*)-20-Hydroxycholesterol	38	50
14	Sitosterol	25-Hydroxycholesterol	40	55

The number of surviving larvae and their mean body weight on day 20, starting with 40 newly hatched larvae, are shown.

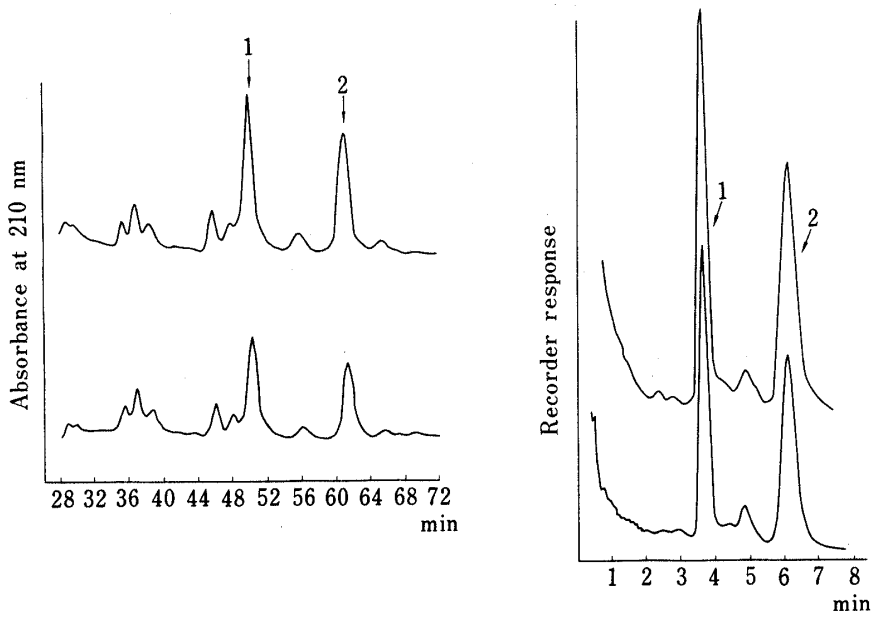

Fig. 3. Silkworm Sterols (Peak 1, Cholesterol; Peak 2, Sitosterol) as Analyzed by HPLC (Left) and by GC-MS in the Form of the TMS Ethers (Right)

Upper: the lipid extract of the larvae reared on 15-oxo-5α-cholest-8(14)-en-3β-ol plus sitosterol. Lower: the lipid extract of the larvae reared on sitosterol. HPLC was run on a reversed phase column of Zorbax ODS (25 cm × 4.6 mm) with methanol at a flow rate of 1.0 ml/min. GC-MS was carried out on a column of 1% OV-17 (1 m × 3 mm) at 270 °C.

In summary, the effect of oxygenated sterols (**1**, **2**, **4**, **7**—**9**), which were added to diet at a much higher concentration than was found in the carcass of *B. cum Botryte*, was too small to induce mortality of *B. mori*. Thus, the death of *B. cum Botryte* is probably not caused by the oxygenated sterols. Our preliminary data indicate that the ratio of triglycerides to sterol fatty acid esters in *B. cum Botryte* is greatly reduced compared to that of *B. mori*. This shortage of the major energy resource, triglycerides, could conceivably be related to the death of the silkworms following fungus infection.

Experimental

Wakogel C-200 was used for column chromatography. Thin layer chromatography (TLC) was carried out on Kieselgel F_{254} plates (0.25 mm thickness, Merck). Compounds on TLC plates were detected by dipping the plates in a 5% ethanolic solution of phosphomolybdic acid, followed by heating at 110 °C. ^{1}H-NMR spectra were obtained with a Hitachi R-24A instrument in $CDCl_3$ solution. A Shimadzu-LKB 9000S apparatus was used for GC-MS. HPLC was performed with a Shimadzu LC-4A apparatus using a ultraviolet (UV) detector (SPD-2AS).

Oxygenated Sterols——Ergosterol peroxide (**1**) was purified from *Bombyx cum Botryte* as described below. 7-Oxocholesterol (**2**) was prepared by oxidation of cholesteryl acetate,[9] followed by saponification with 1% K_2CO_3 in methanol–tetrahydrofuran. A mixture of 7α- and 7β-hydroxycholesterol (**3** and **4**) was prepared by reduction of 7-oxocholesteryl acetate with lithium aluminum hydride, followed by chromatographic separation.[10] 15-Oxo-5α-cholest-8(14)-en-3β-ol (**7**) was prepared by oxidation of 5α-cholest-8(14)-en-3β-ol acetate,[11] followed by saponification with 1% KOH–methanol. (20*S*)-20-Hydroxycholesterol (**8**)[12] and 25-hydroxycholesterol (**9**)[13] were prepared by the cited methods.

Fractionation of Lipid Extract from *Bombyx cum Botryte*——*B. cum Botryte* (250 g) purchased at a market in Shanghai, China, was crushed into small pieces, which were refluxed with 1 l of chloroform–methanol (1 : 1) twice for 2 h. The extract was evaporated under a vacuum to yield 25 g of a residue, which was treated with warm benzene (150 ml). The benzene-soluble portion (10 g) was chromatographed on silica gel (200 g) with a benzene gradient (up to 10%) in ethyl acetate, yielding fraction I (4.5 g, fatty acid esters of sterol and triglyceride), fraction II (3.5 g, free fatty acids), fraction III (0.7 g, free sterols) and fraction IV (0.7 g, free sterols and polar materials). Rechromatography of fraction IV with benzene–ethyl acetate (10 : 1) afforded ergosterol peroxide (110 mg), mp 179—181 °C, NMR: δ 3.6

(1H, m, 3α-H), 5.2 (2H, m, 22, 23-H_2) and 6.21 and 6.50 ppm (2H, ABq, $J=9$ Hz, 6, 7-H_2). For GC-MS of the TMS ether, see Fig. 2. A part (0.3 g) of fraction I was refluxed with a mixture of 30% KOH–methanol (3 ml) and benzene (5 ml) for 2 h to give the saponified fatty acids IF (0.2 g) and the unsaponifiable fraction IS (0.1 g). Parts (*ca.* 2 mg each) of fractions IS and III were treated with TMS imidazole (20 μl) and the resulting sterol TMS ethers were analyzed by GC-MS (for details, see Table I and Fig. 1). Parts (*ca.* 2 mg each) of fractions IF and II were treated with a solution of diazomethane in ethyl ether (0.2 ml), and the resulting fatty acid methyl esters were analyzed by GC-MS using a DEGS column (1 m × 3 mm) at 167 °C. The following fatty acids were identified (% of total fatty acid in fraction IF or II, respectively): palmitic acid (30; 36), stearic acid (1; 1), oleic acid (5; 13), linoleic acid (32; 34) and linolenic acid (31; 15).

Rearing of *Bombyx mori*, and Sterol Analysis——Artificial diet containing one of the oxygenated sterols, **1**, **2**, **4**, and **7**—**9** at 0.1 or 0.01%, together with 0.1% cholesterol or sitosterol, was prepared as described previously.[14] The newly hatched *B. mori* (40 specimens for each group) were reared at 25 °C. On day 20 after hatching, the mean body weights of surviving larvae were recorded (Table II). The lipid extract of larvae was analyzed for sterols by GC-MS as described previously,[15] and by HPLC (for details, see Fig. 3).

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