The Structure of an Inhibitor of Cholesterol Biosynthesis Isolated from Barley*

Asaf A. Qureshi$$, Warren C. Burger$$, David M. Peterson$$, and Charles E. Elson

From the $Cereal Crops Research Unit, Agricultural Research Service, United States Department of Agriculture, Madison, Wisconsin S3705, the 1Department of Agronomy, and the 1Department of Nutritional Sciences, University of Wisconsin, Madison, Wisconsin 53706

Purification of the oily, nonpolar fraction of high protein barley (Hordeum vulgare L.) flour by high pressure liquid chromatography yielded 10 major components, two (I, II) of which were potent inhibitors of cholesterogenesis in vivo and in vitro. The addition of purified inhibitor I (2.5–20 ppm) to chick diets significantly decreased hepatic cholesterogenesis and serum total and low density lipoprotein cholesterol and concomitantly increased lipogenic activity. The high resolution mass spectrometric analysis and measurement of different peaks of inhibitor I gave a molecular ion at m/e 424 (C29H42O2) and main peaks at m/e 205, 203, and 165 corresponding to C15H22O2, C12H26O2, and C12H24O2 moieties, respectively, which are characteristic of d-a-tocotrienol. This identification was confirmed against synthetic samples. The tocotrienols are widely distributed in the plant kingdom and differ from tocopherols (vitamin E) only in three double bonds in the isoprenoid chain which appear to be essential for the inhibition of cholesterogenesis.

It is well established that 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA)$4$ reductase (EC 1.1.1.34) is the first rate-limiting enzyme in the biosynthetic pathways for cholesterol and other isoprenoids (1). There is strong evidence that cholesterol and mevalonic acid (or post-mevalonate, non-sterol products) independently exert feedback regulation on mevalonate biosynthesis (1).

Populations that consume diets rich in cereal grains tend to have a low incidence of cardiovascular disease (2–4). Our studies show that of the cereal grains, barley is the most effective in lowering blood cholesterol levels of experimental animals (5–10). β-D-Glucans, the principal fiber component of barley endosperm might influence cholesterol excretion (11). However, a major cholesterol-suppressive action of barley is at the level of HMG-CoA reductase (5–10). High protein barley flour (HPBF), the commercial pearling fraction consisting of the aleurone, subaleurone, and germ, is the richest source of the cholesterol-suppressive factors (12, 13). Sequential extraction of HPBF with petroleum ether, ethyl acetate, methanol, and water remove all cholesterol-suppressive activity from HPBF; each of the solvent fractions contains HMG-CoA reductase-suppressive material (14). In this report, we describe the isolation of two cholesterol-suppressive metabolites, cholesterol inhibitors I and II, from the petroleum ether-soluble fraction (PESF) of HPBF. The determination of the structure of cholesterol inhibitor I and the results of studies in vivo and in vitro confirming its cholesterol-suppressive action are reported.

EXPERIMENTAL PROCEDURES

Chemicals—Sources of chemicals, substrates, labeled substrates, enzymes, and diagnostic kits were identified previously (12–16). Chemicals and solvents were of analytical grade. HPBF was contributed by the Minnesota Grain Pelting Co., East Grand Forks, MN, and d-a-tocotrienol by Dr. Shiro Urano, Tokyo Metropolitan Institute of Gerontology, Japan (17). The PESF of HPBF was prepared as previously described (14); 100 g of HPBF yields 3.5 g of PESF-HPBF.

Response of Chick and Rat Hepatocytes to PESF-HPBF—Hepatocytes were isolated from livers of fasted (48 h) fed (72 h), 8-week-old white Leghorn pullets and 6-week-od Sprague Dawley male rats (15, 16). The hepatocytes (30 mg of protein) suspended in Krebs-Henseleit buffer (pH 6.8) were incubated with PESF-HPBF (0–10 mg) and 10 μg of Tween 80 in a volume of 1 ml. After 15 min incubation at 37 (rat) or 42 °C (chicken), the incubation mixture was centrifuged at 5000 × g for 2 min at 4 °C. The sedimented cells were suspended in 0.4 ml of homogenizing buffer (0.1 M potassium phosphate, pH 7.4, 4 mM MgCl2, 1 mM EDTA, and 2 mM diithiothreitol) and homogenized with a Polytron homogenizer. Following preliminary centrifugation, the 100,000 × g supernatants (cytosolic fraction) and precipitates (microsomal fraction) were held at −20 °C prior to assay for enzymatic activities. Protein concentrations were determined by a modification of the biuret method using bovine serum albumin as the standard (18).

Fractionation of Cholesterol-suppressive Factors by HPLC—The PESF-HPBF was fractionated by semipreparative HPLC. A 50-μl aliquot of PESF-HPBF (20 mg of PESF-HPBF in methanol: petroleum ether, 2:1) was eluted through a C18 reverse phase column (30 cm × 10 mm inner diameter, 10 μm particle size) with HPLC grade methanol at a flow rate of 1 ml/min. Ten fractions (Fig. 1, detecting wavelength, 205 nm) were collected; pooled fractions were taken to dryness under nitrogen at 5 °C; the dried fractions and Tween 80 were dissolved in sufficient Krebs-Henseleit buffer to provide 2 mg of HPLC fraction and 10 μg of Tween 80/ml. These
FIG. 1. HPLC elution profile of the PESF-HPBF. A 50-μl aliquot of PESF-HPBF (20 mg/ml methanol:petroleum ether, 2:1) was eluted through a C18 reverse phase column (30 cm × 10 mm inner diameter, 10-μm particle size) with HPLC grade methanol at a flow rate of 1 ml/min. Ten fractions (detecting wavelength, 205 nm) were collected. Superimposed are the UV spectra of the fractions which have HMG-CoA reductase-suppressive action. Each peak was scanned using the Perkin-Elmer LC-75 detector with an autocontrol variable wavelength detector.

fractions were tested for in vitro effects on HMG-CoA reductase and fatty acid synthetase activities.

Determination of the Inhibition Potency of HPLC Fractions of PESF-HPBF—Hepatocytes isolated from fasted-refed 12-week-old White Leghorn pullets were incubated with 200 μg of each of the 10 fractions of PESF-HPBF separated by HPLC. The incubation was handled as described above except that it contained 1 μg of Tween 80. The results of this assay indicated that HPLC fractions 5 and 9 contained HMG-CoA reductase-suppressive metabolites. A short term feeding trial confirmed that these fractions carried the cholesterol-suppressive metabolites (results not shown).

Fractionation of PESF-HPBF by Column Chromatography—For large scale purification of the cholesterol inhibitors, 10 g of PESF-HPBF was dissolved in 10 ml of petroleum ether and applied to a 90 × 3.5-cm silicic acid (Bio-Sil A 100–200 mesh, dried at 100 °C for 30 min) column. The sample was eluted with successive applications of petroleum ether:diethyl ether mixtures. Preliminary trials led to the elution scheme described in Fig. 2a. Eleven fractions (A–K) were collected; fractions B and H corresponded to HPLC fractions 9 and 5.

Response of Chicks to PESF-HPBF Chromatographic Fractions—PESF-HPBF (7 g/kg diet) and each of the chromatographic fractions except A of PESF-HPBF (0.5 g/kg diet) were fed to groups of nine 1-day-old broiler chicks for 21 days; the birds were fasted for 48 h and refed for 72 h. Each dietary group was housed in a single brooder with 24 h light and free access to water and diet. The commercial diet consisted of 66.6% ground yellow corn, 25% soybean meal (44% protein), 5% meat scraps (50% protein), 1% alfalfa meal (17% protein), 1% dicalcium phosphate, 0.5% calcium carbonate, and 1% vitamin and mineral mixture. The vitamin and mineral mixture provides per kg diet, 2000 IU of vitamin A, 200 IU vitamin D₃, 10 mg of vitamin E, 5 mg of vitamin K₃, 1.3 g of choline, 1.8 mg of thiamin, 27 mg of niacin, 3.6 mg of riboflavin, 3 mg of pyridoxine, 3 mg of pantothenate, 10 μg of vitamin B₁₂, 2 g of NaCl, 50 mg of ZnSO₄, and 50 mg of MnO₂. Grit (5%) was incorporated into the finished diet. The birds were killed by severing the carotid artery, and blood and liver tissue were collected for analysis. The livers were minced, suspended in the homogenizing buffer (1:2, w/v), and processed as described above. Serum total cholesterol was determined.

Purification of Fractions B and H by HPLC—Column fractions B

### TABLE I

Concentration-dependent suppression of rat and avian hepatocyte (hepatocytes were isolated from livers of fasted-refed animals) HMG-CoA reductase and fatty acid synthetase activities by PESF-HPBF

| PESF-HPBF | HMG-CoA reductase | Fatty acid synthetase |
|---|---|---|
| mg/ml | Chicken | Rat | Chicken | Rat |
| 0 | 19.5 (100) | 17.5 (100) | 17.6 (100) | 17.2 (100) |
| 1 | 18.5 (92) | 16.1 (92) | 15.8 (90) | 16.2 (98) |
| 2 | 15.9 (82) | 14.7 (84) | 14.8 (84) | 15.3 (89) |
| 4 | 15.0 (77) | 14.2 (81) | 12.8 (73) | 14.2 (89) |
| 6 | 14.1 (72) | 13.7 (75) | 12.6 (73) | 13.3 (77) |
| 8 | 13.2 (68) | 13.5 (77) | 11.2 (64) | 12.2 (71) |
| 10 | 12.9 (66) | 13.0 (74) | 10.3 (59) | 11.5 (67) |

* pmol of mevalonic acid synthesized per min/mg of microsomal protein.

### TABLE II

Influence of HPLC-purified components of PESF-HPBF on avian hepatocyte HMG-CoA reductase and fatty acid synthetase activities

| HPLC fraction | HMG-CoA reductase | Fatty acid synthetase |
|---|---|---|
| Control | 75.9 (100) | 63 (100) |
| 1 | 74.2 | 89 |
| 2 | 78.4 | 67 |
| 3 | 86.8 | 71 |
| 4 | 64.9 (66) | 72 |
| 5 | 24.6 (32) | 87 (138) |
| 6 | 74.0 | 61 |
| 7 | 68.9 | 57 |
| 8 | 70.8 | 62 |
| 9 | 31.3 (41) | 42 (67) |
| 10 | 73.7 | 65 |

* pmol of mevalonic acid synthesized per min/mg of microsomal protein.

** Percentage of control activity.
Suppression of Cholesterogenesis by Tocotrienol

FIG. 2. Fractionation of PESF-HPBF by column chromatography. a, 10 g of PESF-HPBF in 10 ml of petroleum ether (PE) was applied to a 90 × 3.5-cm silicic acid (Bio-Sil A 100–200 mesh, dried at 100 °C for 30 min) column. Fractions A and B were eluted with petroleum ether (2500 and 500 ml, respectively); fractions C and D with a 95:5 mixture of petroleum ether:diethyl ether (500 and 1000 ml, respectively); fraction E with 2500 ml of a mixture of 90:10 petroleum ether:diethyl ether; fractions F and G with a 87.5:12.5 mixture of petroleum ether:diethyl ether (1500 and 500 ml, respectively); fractions H and I with a 85:15 mixture of the two solvents (500 and 1500 ml, respectively); fraction J with 2000 ml of a 80:20 mixture; and fraction K with 2000 ml of diethyl ether. b, thin layer chromatography of fractions B, E, and H on Silica Gel G (0.25 mm) with benzene:ethyl alcohol (95:5) after purification by recycling preparative HPLC.

TABLE III

| Treatment | n | Final HMG-CoA reductase | Fatty acid synthetase | Serum cholesterol |
|-----------|---|------------------------|----------------------|------------------|
| Control   | 81 | 359 ± 40* | 188 ± 12* | 174 ± 28* | 154 ± 10* |
| PESF-HPBF | 9  | 381 ± 46 | 142 ± 8* | 146 ± 14* | 111 ± 9* |
| B         | 9  | 350 ± 41 | 149 ± 8* | 124 ± 19* | 128 ± 8* |
| H         | 9  | 356 ± 33 | 134 ± 6* | 215 ± 18* | 125 ± 11* |

*Initial weight, 48 ± 2 g.
*pmol of mevalonate formed per min/mg of microsomal protein.
*nmol of NADPH oxidized per min/mg soluble of protein.
*This grouping includes the control group and all treatment groups for which no treatment effects were noted. Chromatographic fractions were added 0.5 g/kg diet. The chicks were fed for 21 days, fasted for 2 days, and refed for 3 days x ± S.D.
*Final weight of column fraction E treatment group, 394 ± 36 g.
*PESF-HPBF was added 7 g/kg diet.
*Means within a column lacking a common superscript are different (p < 0.01).

Table III shows the effect of PESF-HPBF and of column fractions B and H on hepatic HMG-CoA reductase and fatty acid synthetase activities and serum cholesterol levels of broiler cockerels.

and H were further purified by preparative HPLC and their purity checked by chromatography on a 0.5-mm Silica Gel G plate with a solvent system of 95% benzene, 5% ethanol (Fig. 2b). After plate development and exposure to iodine vapor, examination under UV light revealed a single spot for each fraction.

The separation and testing of the isolated inhibitors were carried out in diffused light. Concentrated fractions were stored at -20 °C and when appropriate, the fractions were dissolved in olive oil.

Concentration-dependent Responses of Hepatocytes to Purified Cholesterol Inhibitor I—Hepatocytes isolated from 10-week-old fasted reed White Leghorn pullets were incubated with 0–100 μg of purified cholesterol inhibitor I (column fraction H) under the conditions described above.

Dose-response of Chicks to Dietary Cholesterol Inhibitor I—Dietary cholesterol inhibitor I (0–20 ppm) was fed to groups of nine 1-day-old broiler chicks for 21 days; the birds were fasted and refed prior to the collection of liver and blood. The remaining conditions of the experiment were identical for those described above except that serum LDL and HDL cholesterol levels were measured. Diets were prepared daily.

Dose-response of Chicks to Intraperitoneally Administered Cholesterol Inhibitor I—White Leghorn cockerels, 4 weeks of age, were divided into six groups of six birds. The birds were fed the diet as described above except that proportions of corn and soybean meal were 61.5% and 30%, respectively. The birds were fasted for 48 h and then refed the diet for 72 h. At the time of refeeding (8:00 am), the control group was given 200 μl of olive oil by intraperitoneal injection. The experimental groups were given intraperitoneal injections of 200 μl of olive oil containing 5, 10, 15, 20, or 25 mg of the purified
Table IV

Suppression of Cholesterogenesis by Tocotrienol

### Hepatocytes were isolated from the liver of a 10-week-old White Leghorn pullet which had been fasted and refed. The incubation period was 15 min. Values are the means of triplicates.

| Cholesterol inhibitor I | HMG-CoA reductase* | Fatty acid synthetase* |
|-------------------------|---------------------|------------------------|
| μM/ml                   | g Mglml             | pmol mevalonic acid    |
|                         |                     | synthesized per min/mg |
| 0                       | 55 (100)            | 72 (100)               |
| 5                       | 45 (87)             | 88 (122)               |
| 10                      | 40 (77)             | 97 (135)               |
| 15                      | 31 (69)             | 112 (156)              |
| 20                      | 30 (57)             | 126 (175)              |
| 25                      | 28 (54)             | 135 (168)              |
| 50                      | 22 (42)             | 148 (188)              |
| 100                     | 23 (44)             | 154 (214)              |

*nmol of mevalonic acid synthesized per min/mg of microsomal protein.

RESULTS

HMG-CoA reductase and fatty acid synthetase were suppressed by PESF-HPBF in a dose-dependent manner in both rat and chick hepatocytes, the response being slightly greater in the latter (Table I). These in vitro assays, the results of which are consistent with in vivo responses to PESF-HPBF (14), provide a rapid means of monitoring the inhibitory potency of the HPLC fractions of PESF-HPBF. Preliminary studies had shown that PESF-HPBF failed to suppress the reductase activity when incubated with preparations of microsomes.

The PESF-HPBF was fractionated by semipreparative HPLC (Fig. 1). Ten crude fractions were collected and the HMG-CoA reductase-suppressive action of each tested in avian hepatocytes. Constituents of fractions 5 and 9 suppressed HMG-CoA reductase (Table II). Although components of both fractions 5 and 9 suppressed HMG-CoA reductase, components of the former enhanced and the latter suppressed fatty acid synthetase. The control activities for both enzymes (Table II) are 3.5 times the activities shown in Table I. Two factors may play roles; the concentration of Tween 80 required for the suspension of PESF-HPBF was 10 times that required for the suspension of the HPLC fractions. Alternatively, hepatocytes used in the second trial were from 12-week-old pullets, whereas those used for the first test were from 8-week-old pullets. In a subsequent experiment, activities determined in hepatocytes from 10-week-old pullets in
inhibitor I inhibited HMG-CoA reductase and enhanced fatty acid synthetase in a dose-dependent manner (Table IV). In vitro HMG-CoA reductase activity decreased linearly in response to 0–15 μg and fatty acid synthetase activity increased linearly in response to 0–25 μg of cholesterol inhibitor I.

The initial identification of cholesterol inhibitor I was based on a high resolution mass spectral analysis which gave a molecular ion at m/e 424 with main fragmentation peaks at m/e 205, 203, and 165 which correspond to molecular formula C_{29}H_{42}O_2 (424.3350), C_{29}H_{38}O_2 (205.1229), C_{29}H_{34}O_2 (203.1882), and C_{29}H_{32}O_2 (165.0913) moieties, respectively (Fig. 4). The major peaks at m/e 205 and 203 are due to the loss of three isoprenoid units (m-219) and additionally, H_2 (m-2). The loss of 40 mass units due to the cleavage of the chroman ring in the molecule gives rise to the peak at m/e 165 (Fig. 5). This pattern is characteristic of the fragmentation pattern of 5-α-tocotrienol (17, 22) and is identical with the fragmentation pattern for synthetic 5-α-tocotrienol which was prepared by Urano et al. (17).

The identification of cholesterol inhibitor I was further strengthened by mass spectral analysis of its acetate derivative (Fig. 6). The mass spectrum gives a molecular ion peak at M* 466 (C_{30}H_{44}O_3) which readily loses 42 mass units (CH_{2}CO) giving rise to an intense peak at m/e 424, the parent compound. Peaks at m/e 247 and 245 represent the loss of three isoprenoid units (m-219) and additionally, H_2 (m-2). Then, loss of CH_{2}CO gives rise to the peaks at m/e 205 and 203; splitting of the chroman ring yields a m/e of 165. The ultraviolet spectrum of cholesterol inhibitor I (λ_{max} 292 nm, Fig. 4) is consistent with that of the synthetic 5-α-tocotrienol and with the values reported for synthetic and natural tocotrienols (17, 22) and with that of HPLC fraction 5 (Fig. 1).

Pure 5-α-tocotrienol is light- and temperature-sensitive. As a constituent PESF of HPBF, the compound is stable for at least 2 weeks. The isolated compound, dissolved in olive oil, was stable for 2 days at room temperature.

The dose-dependent actions of 5-α-tocotrienol isolated from the PESF-HPBF were confirmed by the results of a dietary trial (Table V). Broiler chicks were fed diets containing 0–20 ppm 5-α-tocotrienol for a 3-week period. After a 2-day fast, the chicks were fed 3 days. Diet consumption by pen during the experimental period was 6.3–6.6 kg (±730 g/chick). The dose-dependent lowering of the HMG-CoA reductase activity and serum cholesterol by 5-α-tocotrienol is shown in Table V. The fall in serum cholesterol is primarily in the LDL fraction; the decrease in HDL cholesterol is nonsignificant. Concomitant with the decrease in serum cholesterol is the decrease in cholesterol 7α-hydroxylase activity. As was recorded in the in vitro study (Table IV), 5-α-tocotrienol effected a dose-dependent increase in induced fatty acid synthetase activity.

The protocol for the in vivo studies (Tables III and V) consisted of a 21-day ad lib feeding period, a 2-day period of fasting, and a 3-day period of refeeding the experimental diet to broiler cockerels. In the final study, White Leghorn cockerels which had been fasted for 2 days were administered 5-α-tocotrienol by intraperitoneal injection at 24-h intervals during a 3-day period of refeeding. This protocol produced significant dose-dependent modifications of the hepatic enzyme activities (Table VI). Although the decrease in serum cholesterol was not significant, the level of LDL cholesterol was significantly lowered by this 3-day treatment.

DISCUSSION

The tocotrienols are widely distributed in the plant kingdom. The tocopherols are generally chloroplast components

Fig. 5. Analysis of the mass spectral pattern of cholesterol inhibitor I.
Suppression of Cholesterogenesis by Tocotrienol

The biosynthetic paths are distinguished by the addition of geranylgeranyl pyrophosphate and phytyl pyrophosphate, respectively, to homogentistic acid in production of the tocotrienol and tocopherol metabolites (24). In animals, tocotrienols have 15–25% of the activity of vitamin E (25, 26). The function of the tocotrienols in plants is not known but one role may be as an antioxidant (23). Barley is rich in the various tocotrienols which differ from the tocopherols only in the double bonds of the isoprenoid chain (23). Ubiquinone-9 (27) and possibly squalene (1) suppress HMG-CoA reductase activity. The response of rats to 1.5 mg of ubiquinone-9/day (and ubiquinol-9 acetate) was similar to that recorded for 5 ppm of d-α-tocotrienol. Sterol synthesis was decreased by 40% and fatty acid synthesis increased 8% (27). Tocotrienols, ubiquinone-9, and squalene share in common the isoprenoid chain. This characteristic appears to be essential for the inhibition of cholesterogenesis.

Early in our studies of the feed value of cereal grains, we noted an effect of specific grains on plasma and liver cholesterol concentrations, a pattern of responses that was generally reflected in hepatic HMG-CoA reductase activities in chicks.
fed a cholesterol-free diet. The rank of grains in the order of their increasing effect on the suppression of cholesterol concentrations was: corn (100% or 139 ± 7 mg/100 ml plasma), wheat (76%, 80%), rye (75%, 79%), oats (68%, 75%), and barley (55%, 65%) (6). The tocotrienols as percent of total tocotrienols (mg/kg) are: corn, 23.6; wheat, 23.6; rye, 44.6; oats, 17.8; and barley, 30.5 (28). The tocotrienols as percent of total tocotrienols reflect more closely the measure of the quantity of α-tocotrienol.

The results of this study clearly indicate that dietary α-tocotrienol suppresses hepatic HMG-CoA reductase activity, the first rate-limiting enzyme in the synthesis of cholesterol (3). This suppression of mevalonate biosynthesis results ultimately in the lowering of avian serum LDL cholesterol levels. The results are consistent with the concept that when multiple end products are formed through biosynthetic pathways branching from a common precursor, an end product inhibition by an excess of one product will decrease not only its own production but also that of the products of the other branches (29).

The cholesterol-suppressive value of plant foods (30) may well lie in a broad variety of non-sterol, post-mevalonate products which act in concert with cholesterol derived from LDL in the multivalent regulation of HMG-CoA reductase activity (1).

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TABLE VI
Concentration-dependent effects of α-tocotrienol administered intraperitoneally on cholesterol metabolism of fasted-refed White Leghorn cockerels

| d-α-Tocotrienol | HMG-CoA reductasea | Cholesterol | Fatty acid synthetasea | Serum cholesterol (mg/100 ml) |
|-----------------|-------------------|-------------|-----------------------|-----------------------------|
| mg/day          | Total             | %a-hydroxylyasea | Total | HDL | LDL |
| 0               | 0                 | 35 ± 2a     | 135 ± 13a             | 129 ± 6a                    | 50 ± 8a                       | 63 ± 8a                       |
| 5               | 15                | 31 ± 4a     | 140 ± 14a             | 115 ± 10a                   | 54 ± 7a                       | 49 ± 3a                       |
| 10              | 30                | 25 ± 3a     | 162 ± 14a             | 121 ± 6a                    | 55 ± 8a                       | 48 ± 6a                       |
| 15              | 45                | 22 ± 4a     | 174 ± 13a             | 124 ± 3a                    | 54 ± 6a                       | 47 ± 6a                       |
| 20              | 60                | 21 ± 5a     | 172 ± 15a             | 116 ± 4a                    | 50 ± 5a                       | 42 ± 7a                       |
| 25              | 75                | 22 ± 4a     | 170 ± 10a             | 118 ± 6a                    | 55 ± 7a                       | 50 ± 9a                       |

a nmol of NADPH oxidized per min/mg of cytosolic protein.

 Values not sharing a common superscript letter were different at p < 0.01.