A Fermentation Product of Phytosterol Including Campestenone Reduces Body Fat Storage and Body Weight Gain in Mice

Kunio SUZUKI 1, Rie KONNO 1, Takeshi SHIMIZU 1, Tadashi NAGASHIMA 2 and Akihiko KIMURA 2

1 Technoflora Co. and Synthetic Organic Chemistry Laboratory, RIKEN, Wako, Saitama 351–0198, Japan
2 Toyo Hakko Co., Ltd., 1–39 Yoshikawa-cho, Obu, Aichi 474–0046, Japan

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Summary Anti-obesity effects of a fermentation product of phytosterols including campestenone in ICR mice were investigated. Five-week-old male ICR mice were fed by the pair-feeding method for 8 wk. Experimental feed was prepared by adding TO-001, a phytostenone mixture produced by fermentation of phytosterols using Nocardioides simplex, at 0.25, 0.5, 1.0, or 2.0% or no additive to a high fat diet (fat 20%). Mice fed a stock feed (fat 5.6%) ad libitum were used as the standard growth group. In animals fed the high fat diet, control (no added TO-001) mice showed a weight gain that was about 10% higher than for the standard growth group. TO-001 reduced body weight dose-dependently. Final body weights of 0.5% and 1.0% TO-001-fed mice were lowered by about 9% and those of 2.0% TO-001-fed mice by about 12% compared with the control mice. Visceral and subcutaneous fat weight in mice fed TO-001 was significantly lower than that in mice fed the control diet. The concentrations of serum triglyceride (TG) and total cholesterol (TC) were significantly lower in the 1.0% and/or 2.0% TO-001-fed mice. Furthermore, levels of liver TG and TC were decreased in the TO-001-fed group. Increase of total lipid excretion in the feces was dose dependent. No obvious abnormalities due to consumption of TO-001 were detected by a blood biochemical examination, clinical observations or necropsy. The results suggested that TO-001, a fermentation product of phytosterols, may be a promising component of dietetic functional foods.

Key Words phytostenone, campestenone, fermentation of phytosterol (plant sterol), anti-obesity, visceral fat

Phytosterols, also called plant sterols, are widely distributed in the plant kingdom, and β-sitosterol, campesterol and stigmasterol are found in high concentrations in seeds such as corn, soybeans, sesame and rapeseed, and their oils. Phytosterols are partially metabolized into phytostanols via 3-oxo derivatives by the following pathway (Fig. 1): phytosterol → phytost-5-en-3-one (5-phytostenone) → phytost-4-en-3-one (4-phytostenone) → 5-phytostan-3-one (phytostanone) → 5-phytostanol (phytostanol). This metabolic pathway is reported in higher plants, and the metabolic intermediates and phytostanol are usually distributed only in trace amounts in plants (1–3).

Phytosterol inhibits the uptake of dietary cholesterol from the small intestine through competition for the micellar solubility of cholesterol, causing a decrease in blood cholesterol levels. Sugano et al. (4) reported that phytostanol, a saturated metabolite of phytosterol, exhibits hypocholesterolemic action much greater than that of the original phytosterol.

On the other hand, Konno et al. (5) found that phytosterones, particularly in 5-campestenone, have anti-obesity action by reducing blood TG and body fat accumulation, in addition to their hypocholesterolemic activity. They also reported that this compound acts as an anti-diabetic in the type 2 diabetes animal models, C57BL/Ksj-db/db mice (6) and Zucker diabetic fatty rats (7).

The mode of action of campestenone was recently clarified as inhibition of chylomicron biosynthesis in the small intestine and acceleration of mRNA expression of the enzyme groups concerned in fatty acid β-oxidation in the liver (8). The mRNA expression is thought to be closely related to activation of the nuclear transcription factor, peroxisome proliferator-activated receptor alpha (PPARα).

In an attempt to produce campestenone by the fermentation of phytosterol, we developed a two-layer fermentation method using Nocardioides simplex, and obtained a phytostenone mixture, TO-001, including campestenone (9).

The aim of this study was to examine the effect of dietary TO-001 on lipid metabolism and body fat accumulation in mice, and to investigate its possible use as a functional food.

MATERIALS AND METHODS

Materials. Phytostenone mixture TO-001 was produced by two-layer fermentation of phytosterol using Nocardioides simplex developed by Suzuki et al. (9). As
shown in Table 1, TO-001 contained approximately 16% campestenone, 29% \( \beta \)-sitostenone, 3% phytost-4-en-3,6-dione and 52% phytosterol.

**Laboratory animals and group assignment.** Fifty-four male ICR mice, 5 wk old, obtained from Charles River Japan Inc. (Kanagawa) were divided into 6 groups (9 mice each) as follows after a 1-wk feeding with stock feed.

1) Stock feed group for standard growth
2) High-fat diet control group for experimental diets
3) 0.25% TO-001-added high-fat diet group
4) 0.5% TO-001-added high-fat diet group
5) 1.0%TO-001-added high fat diet group
6) 2.0%TO-001-added high-fat diet group

**Feed.** The stock feed group were given commercial feed (type CRF-1, Oriental Yeast Co., Ltd., Tokyo; protein, 22.6%; fat, 5.6%; nitrogen-free extract, 53.8%; calories, 1,490 kJ/100 g) ad libitum and the other groups were pair-fed each experimental diet. The basal experimental diet was a semipurified diet (modified AIN, Oriental Yeast Co., Ltd.) that contained casein, 20%; fat (lard), 20%; cornstarch, 38%; \( \alpha \)-cornstarch, 8%; sucrose, 4%; cellulose, 6.4%; vitamin mixture, 0.8%; mineral mixture, 2.8% with calories of 1,925 kJ/100 g.

In order to exclude any effects from differences in food intake, feed consumption of group 6 (2.0% TO-001), the lowest among all groups, was weighed every day; and the same amounts of feed were given to the other groups except for group 1 (stock feed) by the pair-feeding method.

**Housing conditions.** Mice were housed in a standard plastic cage with 4–5 animals per cage and maintained at 24±1°C and 50±5% relative humidity with a 12 h-light/12 h-dark cycle. Bedding was paper Tek-Fresh (Edstron Japan, Tokyo). Cages and bedding were changed once a week. The experimental diet was prepared every 2 wk and stored at 4°C before use. Animal care and the experiments were carried out in accordance with the guidelines for animal experimentation of RIKEN and the Japanese Association for Laboratory Animal Science.

**Measurement methods.** Body weights of the animals were determined twice a week and feed intake was calculated from the daily feed residue. On completion of feeding, the animals were sacrificed without starvation by exsanguination from the vena cava under carbon dioxide inhalation. The brain, lung, heart, liver, kidneys, spleen, testes, adrenal glands, visceral adipose tissue (perirenal, mesenteric and periepididymal), abdominal subcutaneous adipose tissue and brown adipose tissue were excised and weighed.

Serum lipids were measured by enzyme assay using an autoanalyzer (Mitsubishi Kagaku Bio-Chemical Laboratories, Inc., Tokyo). Feces were collected for the last 2 d of feeding and weighed. Fecal lipids were extracted by the method of Jeejeebhoy (10). Liver lipids were extracted by the method of Folch et al. (11). Each lipid component of fecal and liver lipids was measured with commercial kits (Triglyceride E test, Cholesterol C test, NEFA C test, Phospholipid C test, and Total bile acid test, Wako Pure Chemical Industries, Ltd., Osaka).

**Statistical analysis.** Data were subjected to statistical analysis using two-way analysis of variance (ANOVA) for the time-course of body weight (Fig. 1) and one-way ANOVA with pairwise comparison by the Bonferroni method for growth, body fat accumulation and lipid concentration in serum and liver (Table 2) and the blood biochemical profile (Table 4).

**RESULTS**

**Body weight**

Changes in body weight in each group are shown in Fig. 2. High-fat control mice (Group 2) showed rapid body weight gain that was about 10% higher than the standard growth curve of the stock feed group (Group 1). At 28 d of age, clear weight loss was observed in the TO-001-fed group, and differences in body weight were

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**Table 1. Composition of TO-001.**

| Component                  | %   |
|----------------------------|-----|
| Campest-5-en-1-one         | 3   |
| Campest-4-en-1-one         | 13  |
| \( \beta \)-Sitost-5-en-1-one | 6   |
| \( \beta \)-Sitost-4-en-1-one | 23  |
| Phytost-4-en-3,6-dione     | 3   |
| Phytosterols               | 52  |
gradually enhanced during the feeding period. The variance among values in the experimental groups except for the 0.25% TO-001 group was statistically significant (p<0.001).

Final body weight of TO-001-fed mice ranged from 97% (Group 3, 0.25%-added feed) to 88% (Group 6, 2.0%-added feed) of those of the control mice. Body weights of 0.5% TO-001-fed mice (Group 4) were similar to those of 1.0% TO-001 fed mice (Group 5), and 90% of that of the control mice (Group 2) on the last day. After 32 d of feeding, the body weight of 2.0% TO-001-fed mice (Group 6) became lower than that of the standard growth group (Group 1).

Body weight gains for 56 d of feeding were significantly lower in the 0.5% TO-001 groups than that in the control group (Table 2).

**Body fat weight**

As shown in Table 2, visceral and subcutaneous fat weight in mice fed TO-001 was significantly lower than that in those fed the control diet (Group 2). Moderate dose dependency was observed for this reducing effect on fat deposition. On the other hand, brown fat weight showed no significant differences among the groups.

**Serum and liver lipids**

The concentrations of serum TG were significantly lower in the 1.0% or 2.0% TO-001-fed mice, but not in the 0.25% or 0.5% TO-001 groups. A significantly low concentration of TC was observed only in the 2.0% TO-001-fed mice (Table 2). A decreasing tendency in the levels of liver TG was observed in the TO-001-fed group, especially in 2.0%-TO-001-fed mice. The levels of liver TC were markedly decreased in all TO-001-fed groups (Table 2).

**Fecal lipids**

Dose response for the excretion of fecal lipid is shown in Table 3. A proportional increase of fecal excretion of total lipid and each lipid component was observed for all TO-001 doses.

**Blood biochemical profile**

Blood glucose and Ca levels of 1.0% and 2.0% TO-

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### Table 2. Effect of dietary TO-001 on growth, body fat accumulation and lipid concentration in serum and liver of mice.

|                          | Stock feed | Control | 0.25% | 0.50% | 1.00% | 2.00% |
|--------------------------|------------|---------|-------|-------|-------|-------|
| Initial body weight (g)  | 30.1±0.2   | 31.6±0.4| 31.5±0.5| 31.2±0.5| 31.8±0.6| 31.5±0.6|
| Final body weight (g)    | 39.4±1.0   | 43.3±1.0| 41.3±1.0| 39.5±1.1| 39.7±1.4| 38.2±1.7*|
| Body weight gain (g)     | 9.35±0.92  | 11.69±1.00| 10.74±1.00| 8.30±0.72| 7.94±1.12*| 6.84±1.50*|
| Visceral fat weight (g)  |            |         |       |       |       |       |
| Perirenal                | 0.44±0.06* | 0.85±0.06| 0.55±0.07*| 0.54±0.09*| 0.54±0.10*| 0.44±0.07*|
| Epididymal               | 1.12±0.10* | 2.10±0.15| 1.70±0.22| 1.50±0.18| 1.63±0.23| 1.40±0.22 |
| Mesenteric               | 0.45±0.06* | 0.77±0.07| 0.67±0.09| 0.47±0.07*| 0.49±0.07*| 0.49±0.07*|
| Subcutaneous fat weight (g)* | 0.25±0.02*| 0.40±0.02| 0.30±0.03*| 0.30±0.03*| 0.28±0.03*| 0.26±0.03*|
| Brown fat weight (g)     | 0.23±0.02  | 0.27±0.03| 0.20±0.02| 0.26±0.03| 0.26±0.02| 0.24±0.02 |
| Serum lipid concentration (mg/dL) |          |         |       |       |       |       |
| Triglyceride (mg/dL)     | 117.4±10.4*| 57.3±9.6| 37.6±5.8| 42.4±7.6| 29.7±3.9*| 28.8±2.8*|
| Total cholesterol (mg/dL)| 139.7±6.0 | 162.3±8.9| 154.9±4.3| 143.0±10.4| 145.0±8.6| 129.8±8.6*|
| Liver lipid concentration (mg/g) |        |         |       |       |       |       |
| Total lipids             | 69.6±10.7* | 122.5±13.7| 80.0±5.7*| 77.6±5.4*| 88.2±7.9*| 56.5±3.8*|
| Triglyceride             | 34.6±10.2* | 75.4±11.6| 47.1±6.8| 47.6±5.6| 56.9±8.6| 23.1±2.5*|
| Total cholesterol        | 4.7±1.4*  | 10.8±2.0| 3.5±0.2*| 3.7±0.2*| 4.8±0.5*| 2.3±0.1* |

*Abdominal area.
Value represents mean±SE of 9 mice.
Mice were fed a diet with or without TO-001 for 8 wk.
* Significant difference from the control group at p<0.05.
Table 3. Effect of dietary TO-001 on fecal excretion of lipids in mice.

|                          | Stock feed | Control | 0.25%       | 0.50%       | 1.00%       | 2.00%       |
|--------------------------|------------|---------|-------------|-------------|-------------|-------------|
| Food intake (g/d/mouse)  | 6.3        | 4.4     | 4.4         | 4.4         | 4.4         | 4.4         |
| Fat intake (mg/d/mouse)  | 353        | 880     | 880         | 880         | 880         | 880         |
| Feces excretion (mg/d/mouse) | 492.42    | 381.77  | 387.78      | 370.71      | 498.03      | 506.99      |
| Fecal lipids (mg/g of feces) | 15.53     | 32.71   | 39.46       | 49.45       | 70.83       | 99.22       |
| Total lipid excretion (mg/d/mouse) | 7.65      | 12.49   | 15.3        | 18.33       | 35.28       | 50.31       |
| Triglyceride (mg/d/mouse) | 0.58       | 0.72    | 0.65        | 0.79        | 0.91        | 2.35        |
| Total cholesterol (mg/d/mouse) | 0.13     | 0.2     | 0.74        | 1.21        | 3.66        | 4.35        |
| NEFA (mg/d/mouse)        | 4.52       | 3.47    | 3.31        | 3.13        | 5.81        | 6.14        |
| Phospholipids (mg/d/mouse) | 1.77     | 1.61    | 1.29        | 1.19        | 3.67        | 9.22        |
| Total bile acids (mg/d/mouse) | 0.01    | 0.01    | 0.04        | 0.08        | 0.13        | 0.25        |
| Others (mg/d/mouse)      | 0.64       | 6.48    | 9.27        | 11.93       | 21.1        | 28.0        |

Mice were fed a diet with or without TO-001 for 8 wk.

Table 4. Blood biochemical profile of mice fed TO-001.

|                          | Stock feed | Control | 0.25%       | 0.50%       | 1.00%       | 2.00%       |
|--------------------------|------------|---------|-------------|-------------|-------------|-------------|
| TP (g/dL)                | 5.34±0.07  | 5.30±0.08| 5.03±0.06   | 5.44±0.12   | 5.41±0.05   | 5.08±0.10   |
| Alb (g/dL)               | 2.83±0.04  | 2.80±0.07| 2.71±0.03   | 2.85±0.08   | 2.96±0.03   | 2.75±0.06   |
| A/G                      | 1.14±0.02  | 1.14±0.03| 1.16±0.02   | 1.10±0.03   | 1.22±0.01   | 1.20±0.03   |
| CK (IU/L)                | 179.29±71.78| 97.44±26.98| 40.38±7.27 | 84.75±9.56 | 153.33±31.77| 91.50±30.96|
| GOT (IU/L)               | 71.71±10.28| 60.89±5.99 | 43.13±1.96 | 62.0±6.01 | 74.0±9.40 | 59.25±10.48 |
| GPT (IU/L)               | 25.0±4.40  | 38.56±7.16| 22.38±2.04 | 39.38±8.12 | 28.11±2.76 | 20.88±2.24  |
| ALP (IU/L)               | 158.43±10.61| 129.33±11.56| 155.63±13.54| 182.75±15.72| 231.89±31.46*| 138.0±14.81|
| γ-GTP (IU/L)             | 1.0±0.0    | 1.0±0.0  | 1.0±0.0     | 1.0±0.0     | 1.0±0.0     | 1.0±0.0     |
| Creatinine (mg/dL)       | 0.17±0.01  | 0.19±0.01| 0.20±0.01   | 0.20±0.01   | 0.20±0.01   | 0.20±0.02   |
| BUN (mg/dL)              | 25.29±1.44*| 15.0±0.58| 16.88±0.67  | 17.50±0.78  | 21.56±1.48*| 21.75±1.35*|
| Bilirubin (mg/dL)        | 0.11±0.01  | 0.11±0.01| 0.10±0.0    | 0.10±0.0    | 0.13±0.02   | 0.13±0.02   |
| Glucose (mg/dL)          | 227.57±13.54| 268.33±20.75| 293.25±16.74| 226.0±10.74| 135.22±23.95*| 145.88±17.72*|
| Na (mEq/L)               | 156.0±0.49 | 153.11±0.59 | 151.38±0.50 | 153.38±0.65 | 154.44±0.65 | 152.88±0.67 |
| K (mEq/L)                | 9.49±0.34  | 10.51±0.42| 10.89±0.44  | 10.31±0.36  | 11.23±0.50  | 12.25±0.15* |
| Cl (mEq/l)               | 103.29±0.75| 105.44±0.47 | 107.25±0.56 | 103.13±1.06| 106.11±0.31 | 110.38±0.71 |
| Ca (mg/dL)               | 9.44±0.36  | 9.86±0.13 | 9.63±0.09   | 9.68±0.31   | 9.0±0.20*   | 8.81±0.20*  |
| P (mg/dL)                | 15.21±0.31 | 12.92±0.58| 9.38±0.34   | 16.19±0.76  | 13.34±0.53  | 11.68±0.46  |

* Significant difference from the control group at p<0.05.
Value represents mean±SE of 9 mice.

Mice were fed a diet with or without TO-001 for 8 wk.

001-fed mice were significantly decreased but were within the normal range (Table 4). The blood potassium level of the 2.0% TO-001-fed group was slightly (1.2 times) but significantly higher than that of the control mice (Group 2). Blood urea nitrogen (BUN) levels of 1.0% and 2.0% TO-001 fed mice were significantly increased but were within the normal range. No significant difference due to consumption of TO-001 was detected in the levels of total protein (TP), albumin, albumin/globulin ratio (A/G), creatinine kinase (CK), alkaline phosphatase (ALP), alanine aminotransferase (GPT), aspartate aminotransaminase (GOT), γ-glutamyl transpeptidase (γ-GTP), creatinine, bilirubin, Na, Cl or P.

General signs and necropsy findings

All animals were healthy without any clinical abnormalities. No pathological anomaly in the organs due to consumption of TO-001 was observed in necropsy except for liver weight in the 2.0% dose group (Group 6), which was 0.9 times lower than that of the controls.

DISCUSSION

Phytostenone mixture TO-001 produced by fermentation of phytosterol with Nocardiaoides simplex showed a...
clear dose-dependent effect on body-weight loss without any anomalies in the necropsy or blood biochemical profile of ICR mice.

The content of phytostenone in TO-001 was about 50%, and 5-en compounds such as sitost-5-en-3-one and campest-5-en-3-one accounted for 9.3%, 4-en compounds such as sitost-4-en-3-one and campest-4-en-3-one for 36.2%, and 3,6-dione compounds for 3.0%. Konno et al. reported that dietary exposure to these phytostenones, particularly campest-5-en-3-one and sitost-4-en-3, 6-dione, had potent reducing effects on body weight, abdominal fat weight and serum lipid concentration in CDF1 mice. However, the weight-loss effect of TO-001 was potent even though the levels of campest-5-en-3-one and 3,6-dione compound were comparatively low. It seems probable that each phytostenone in TO-001 has additive but not competitive effects as a weight-loss agent.

Body fat weight of TO-001-fed mice was significantly decreased for both visceral and subcutaneous fat with a slight dose dependency, but not for brown adipose tissue. Furthermore, dietary TO-001 reduced hepatic and blood TG and TC. We observed an anti-obesity effect based on campest-5-en-3-one in Sprague-Dawley rats. According to our observations, the compound possesses ligand affinity to the nuclear receptor PPARα and accelerates β-oxidation of fatty acid in the liver. Our results suggest that all phytostenones in TO-001 act as agonists for PPARα because of the similarities in physiological activity and chemical structure to campest-5-en-3-one.

We also demonstrated that fecal excretion of lipid was increased by the addition of TO-001, suggesting that phytostenones inhibited intestinal absorption of fat. However, phytosterols and phytostenones that originate in the experimental feed are present in the total excretion of lipids, and the concentration of major lipid fractions such as triglyceride and free fatty-acid (NEFA) was less than 1% of fat intake. Therefore, it appeared that the reduction of fat absorption is not a major cause of the decrease of body weight or body fat accumulation in the animals fed TO-001.

In the blood biochemical analysis, blood glucose levels in the high-dose group of TO-001 were significantly decreased at 1.0% and 2.0%, Phytostenones in TO-001 may have anti-diabetic action in the same way as campest-5-en-3-one, which is the same 3-oxo derivative and causes hypoglycemia in C57BL/Ksj-db/db mice (11) and Zucker diabetic fatty (ZDF) rats (6) as animal models of type 2 diabetes. Since the blood biochemical profile, clinical observations and necropsy generally showed little change after consumption of TO-001 for 8 wk, it appeared that TO-001 had very weak toxicity in test animals.

Our results clearly showed that TO-001, a fermentation product of plant sterols, prevents body fat deposition and diet-induced obesity by dietary supplementation.

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REFERENCES

1) Akihisa T, Kimura Y, Roy K, Ghosh P, Thakur S, Tamura T. 1994. Triterpene alcohols and 3-oxo steroids of nine leguminosae seeds. Phytochemistry 35: 1109–1113.
2) Fernandez MI, Pedro JR, Seoane E. 1983. Constituents of a hexane extract of Phoenix dactylifera. Phytochemistry 22: 2087–2088.
3) Gaspar, EMM, Das Neves, HJC. 1993. Steroidal constituents from mature wheat straw. Phytochemistry 34: 523–527.
4) Sugano M, Morioka H, Ikeda I. 1977. A comparison of hypcholesterolemic activity of β-sitosterol and β-sitostanol in rats. J Nutr 107: 2109–2112.
5) Konno R, Suzuki K, Hasegawa K. 2000. Lowering effect of phytostenone on serum lipid concentration and body fat accumulation, and its mechanism in mice. Annal J Nutr Sci Kagawa Nutr Univ 8: 45–54.
6) Suzuki K, Tanaka M, Konno R, Kaneko Y. 2002. Effect of 5-campestenone (24-methylcholest-5-en-3-one) on the type 2 diabetes mellitus model animal C57BL/KsJ-db/db mice. Horm Metab Res 34: 121–126.
7) Konno R, Kaneko Y, Suzuki K, Matsui Y. 2005. Effect of 5-campestenone (24-methylcholest-5-en-3-one) on Zucker diabetic fatty rats as a type 2 diabetes mellitus model. Horm Metab Res 37: 79–83.
8) Ikeda I, Konno R, Shimizu T, Ide T, Takahashi N, Kawada T, Nagao K, Inoue N, Yanaugita T, Hamada T, Morinaga Y, Tomoyori H, Imaizumi K, Suzuki K. 2006. Campest-5-en-3-one, an oxidized derivative of campesterol, activates PPARα, promotes energy consumption and reduces visceral fat deposition in rats. Biochim Biophys Acta 1760: 800–807.
9) Suzuki K, Nagashima T, Nagahashi S. 2004. Production method of 5-en-3-one or 3,6-dione compound of sterol, and the method of production and analysis of lipid-metabolism-improving material, food and animal feed. Japanese Patent Toku-gan 2004-207885.
10) Jeejeebhoy KN, Ahmad S, Kozak G. 1970. Determination of fecal fats containing both medium and long chain triglycerides and fatty acids. Clin Biochem 3: 157–163.
11) Folch J, Lees M, Sloane Stanley GH. 1957. A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 226: 497–509.