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NOTE

Anti-obesity activities of the yoshinone A and the related marine γ-pyrone compounds

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Marine cyanobacteria are known as important creators of novel natural products. From this valuable source, various bioactive compounds have been found and characterized in terms of their pharmacological and toxicological activities.[1] In the previous work, we have reported on the isolation and structure determination of potent cytotoxic compounds, lyngbyacyclamide A and B;[2] an inhibitor of osteoclastogenesis, biselyngbyaside;[3] and a protein kinase inhibitor, bisebromoamide.[4] In the recent work, we have reported the new marine γ-pyrones yoshinone A, B1 and B2 from *Leptolyngbya* sp., and determined their planar structures using NMR spectral analysis.[5] Yoshinone A, as the major compound among them, showed inhibitory activity against the adipogenic differentiation of 3T3-L1 cells with an half maximal inhibitory concentration (IC_{50}) value of 420 nM without cytotoxicity ($IC_{50} > 50$ μM). On the other hand, the yoshinone B1 and B2 showed only limited activity against 3T3-L1 cells, with higher concentrations compared with yoshinone A. Further studies of the structure–activity relationship lead us to conclude that the position of a pyrone ring and an olefin in the side chain will be important for the inhibition of adipogenic differentiation. These γ-pyrones have olefins in their side chain at positions 7 and 6 in the cases of yoshinones A and B1/B2, respectively. To express the effects on adipocyte, the olefin should not be conjugated with γ-pyrone moiety, such as yoshinone A (Figure 1). In the previous studies, kalkipyrone[6] isolated from cyanobacteria, aureothin,[7] and actinopyrones A and B[8] isolated from streptomyces fell into the same 7-en γ-pyrones. Then, we confirmed that kalkipyrone and aureothin showed this activity, with IC_{50} values of 67.5 and 54.2 nM, respectively. On the basis of these data, we are focusing on the 7-en γ-pyrone (unconjugated type) compounds. These pyrones are expected to be candidates for novel lead compounds for the treatment of obesity and related diseases.[9] Studies on useful tools that regulate adipocytes will contribute to the prevention and treatment of these diseases. At the present stage of our research, we have evaluated the anti-obesity activities of the 7-en γ-pyrones using *in vitro* and *in vivo* experiments. In this study, we report on the interesting properties of these pyrones.

Marine cyanobacteria as sources of γ-pyrone compounds have been collected from Ishigaki and Okinawa islands, Japan, and extracted with aqueous methanol. The isolating procedures of the γ-pyrones were performed according to the original reports with minor modifications. Their purity and structures were confirmed by NMR analysis. From the collected cyanobacteria, we noted that got only a trace amount of yoshinone A (<1.0 mg) and 35.7 mg of kalkipyrone, as purified γ-pyrones for the present experiments.

As the *in vitro* experiments, the reducing effects on accumulated triglyceride (TG) in the mature 3T3-L1 adipocyte were investigated with yoshinone A. In Figure 2a, typical images of mature 3T3-L1 adipocytes stained TG with oil red O were shown. As shown in Figure 2b, TG amount in mature 3T3-L1 adipocyte-treated yoshinone A significantly decreased with the dose-dependent manner. On the other hand, a significant increase of lactate (LA) in the culture fluid was observed by yoshinone A treatment of the adipocyte, as shown in Figure 2c. These changes in TG and LA were induced with 0.1–0.01 μM of yoshinone A with no effect on cell viability. These results revealed that the 7-en γ-pyrones showed TG reduction activities in mature 3T3-L1 adipocyte, in addition to its inhibitory activities on adipose differentiation. As the differentiation ratio in preadipocyte was evaluated with TG amount in the cells, the findings are acceptable for the previous experimental perceptions in 3T3-L1 cells.

In the following experiment, to verify whether or not the LA production is limited in 3T3-L1 cells, we have repeated the experiment with the same conditions using HeLa cells, the most widely used human cultured cells. After treatment with yoshinone A (0, 0.01 and 0.1 μM) for 48 h, the culture fluids were supplied for HPLC analysis to

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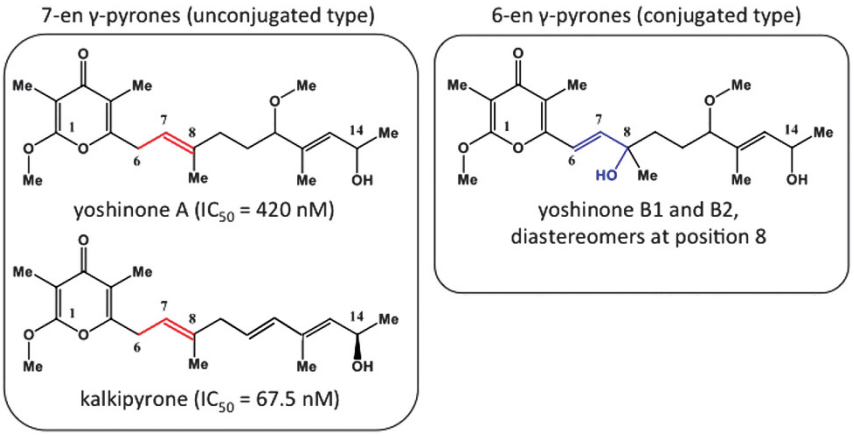

Figure 1 Chemical structures and biological activities of the two types of marine γ-pyrones. The 7-en γ-pyrone is defined as an unconjugated olefin bond (red) at 7 position in the side chain such as yoshinone A and kalkipyrone, and the 6-en γ-pyrone is defined as a conjugated olefin bond (blue) at 6 position in the side chain such as yoshinone B1 and B2. Inhibitory activities against adipogenic differentiation in 3T3-L1 cells were expressed as half maximal inhibitory concentration (IC_{50}) values in the parenthesis. The yoshinone B1 and B2 showed only moderate inhibition even at 5 μM.

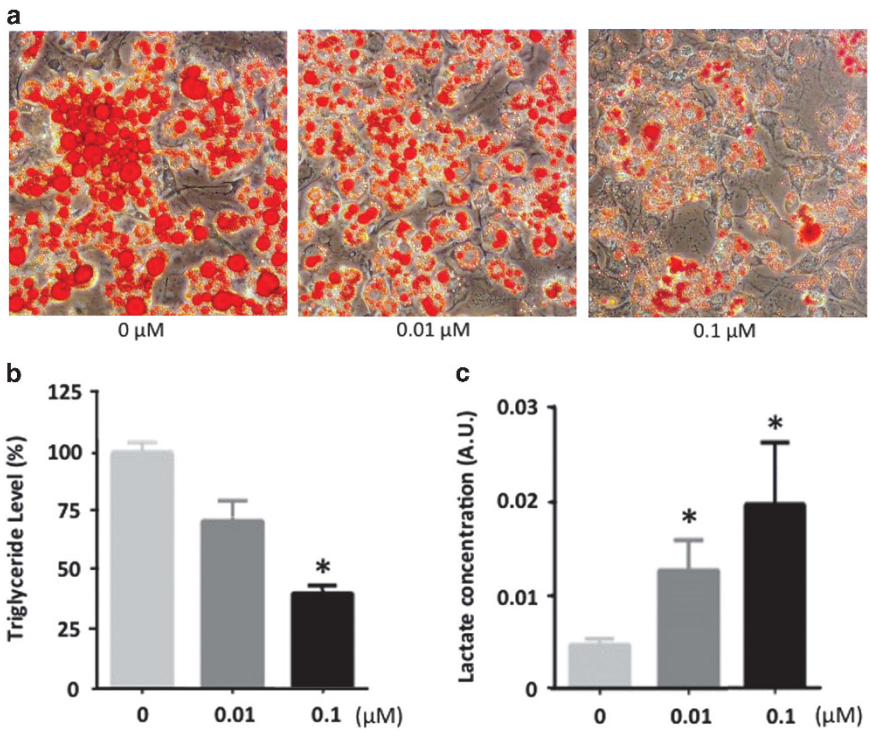

Figure 2 The decrease of accumulated triglyceride in 3T3-L1 cells and enhancement of lactate contents in culture fluid treated with yoshinone A (0, 0.01 and 0.1 μM). The mature 3T3-L1 adipocytes were treated with yoshinone A for 7 days. Typical adipocytes were stained with oil red O (**a**), the % of triglyceride level in the cells (**b**) and lactate concentration in the culture fluid after 48 h (**c**) were shown. Data are presented as the mean ± s.d. for triplicate samples. *$P<0.05$ vs control (0 μM).

determined LA and glucose (Glc) concentrations. As shown in Supplementary Figure 1, the enhancement of LA production and Glc consumption were observed clearly with dose-dependent manner of yoshinone A even in HeLa cells without cytotoxicities. These results suggested that yoshinone A induced energy metabolic changes relating to Glc via relatively common pathway in cells. On the basis of these *in vitro* experiments, the induced changes in metabolism will affect the utilization of accumulated TG in mature 3T3-L1 adipocyte with direct or indirect pathway(s). Due to a deficiency of these marine γ-pyrones, further experiments to analyze the mechanism in detail are still needed at this stage.

As with the *in vivo* experiments, kalkipyrone as a 7-en γ-pyrone was provided to the experiment with a small preliminary sample size. To confirm the anti-obesity activity *in vivo*, we performed two experiments as described below.

The IC_{50} value of kalkipyrone was reported as 120 nM in HeLa cells,[5] but there are no information about the toxicity *in vivo*. In the first experiment using mice, the evaluation for acute toxicity of kalkipyrone with oral administration was determined. Male ddY mice (5 weeks old) were divided into three groups ($n=3$ each); they received 16.5 and 5.5 mg kg^{-1} per day of kalkipyrone, and vehicle (3% dimethyl sulfoxide solution) orally for 3 days. The physical

measurements, autopsy findings and behavior observations did not indicate a difference among these groups during 7 days from first ingestion. Then, we planned a long-period treatment test in mice with a dosage of 5 mg kg^{-1} per day of kalkipyrone.

For the second experiment *in vivo*, the anti-obesity effects of kalkipyrone *in vivo* were examined by feeding mice a high-fat diet (HFD) for 5 weeks. Mice were fed a normal diet (ND, $n=6$), a HFD ($n=6$) and HFD with oral ingestion of kalkipyrone at a dosage of 5 mg kg^{-1} per day (HFD+KAL, $n=3$) during the experiment. The transitional changes of body weight gain, food intake and water intake of the groups are shown in Supplementary Figure 2. After the experimental period, measured parameters were summarized in Table 1. The body weights of mice in the ND and HFD groups showed significant differences, with the values of 39.5 ± 0.2 and 43.4 ± 0.7 g, respectively. The HFD+KAL group (40.6 ± 2.8 g) exhibited pronounced suppressed body weight gain, but no significant differences, owing to the limited sample size for the experiment. Meanwhile, the weight of adipose tissue was significantly suppressed ($P<0.05$) with the kalkipyrone treatment: 0.93 ± 0.23 g in the HFD +KAL group vs 1.62 ± 0.15 g in the HFD group. The other tissues' weight did not show significant changes with kalkipyrone treatment (Supplementary Figure 3). These results suggest that oral ingestion of kalkipyrone is effective for suppressing adipose tissue weight gain in mice.

As food intake during the experimental period in the HFD (227.9 ± 19.3 g per head) and HFD+KAL (212.8 ± 31.3 g per head) groups were similar, the suppression of adipose tissue gain was not affected by food consumption or appetite in mice. Another main possible mechanism for anti-obesity is the inhibition of TG absorption in the small intestine. For example, orlistat, a lipase inhibitor in clinical use,[10] suppresses the weight gain *in vivo* to avoid absorption of TG from small intestine. Also, tea polyphenol reduces fat absorption by decreasing emulsification,[11] and hot-water extract of *Houttuynia cordata* suppressed adipose tissue weight gain in mice by inhibiting the absorption of fatty acids and glycerol.[12] These suppressive effects on TG absorption will result in the intact TG undergoing a transition to feces, accompanied increasing fecal TG levels. However, fecal TG in the HFD+KAL group (43.6 ± 6.0 mg g^{-1}) was not increased, as compared with that in the HFD group (41.3 ± 7.4 mg g^{-1}). Thus, it was suggested that the suppression of adipose tissue gain by kalkipyrone treatment is caused by consumption and/or excretion of absorbed TG in the body.

Table 1 Effects of kalkipyrone on high-fat diet received mice for 5 weeks

	Experimental groups		
Measured parameter	*ND* (n = *6*)	*HFD* (n = *6*)	*HFD+KAL* (n = *3*)
Body weight (g)	39.5 ± 0.2	43.4 ± 0.7	40.6 ± 2.8
Food intake (g)[a]	168.2 ± 4.1	227.9 ± 19.3	212.8 ± 31.3
Water intake (g)[a]	205.1 ± 7.1	192.0 ± 9.5	194.3 ± 14.7
Adipose tissue (g)	1.01 ± 0.12	1.62 ± 0.15	0.93 ± 0.23*
Liver (g)	1.31 ± 0.03	1.41 ± 0.07	1.35 ± 0.11
Hepatic TG (mg g^{-1} liver)	38.2 ± 6.0	41.3 ± 5.7	34.7 ± 5.8
Feces (g)[b]	1.4 ± 0.2	2.5 ± 0.6	1.8 ± 0.2
Fecal TG (mg g^{-1} feces)	9.8 ± 0.2	41.3 ± 7.4	43.6 ± 6.0
Plasma Glc (mg dl^{-1})	133.4 ± 13.7	135.7 ± 16.6	155.1 ± 28.2
Plasma LA (mg dl^{-1})	65.6 ± 6.1	68.8 ± 6.1	95.4 ± 2.4*
Plasma TG (mg dl^{-1})	134.5 ± 34.5	132.1 ± 25.5	88.0 ± 16.1
Plasma TC (mg dl^{-1})	159.3 ± 18.3	141.2 ± 22.0	130.0 ± 31.5
Plasma NEFA (mEq l^{-1})	0.88 ± 0.17	0.74 ± 0.06	0.70 ± 0.10

Abbreviations: ANOVA, analysis of variance; Glc, glucose; HFD, high-fat diet; HFD+KAL, high-fat diet with kalkipyrone (5 mg kg^{-1} per day per os); LA, lactate; ND, normal diet; NEFA, non-esterfied fatty acid; TC, total cholesterol; TG, triglyceride.
[a]Accumulated values during experiments.
[b]Total values for 3 days.
*$P<0.05$ vs HFD.
Data are presented as the mean ± s.e. and analyzed by ANOVA followed by Dunnett's test.

Some changes in plasma parameters were induced by the treatment of kalkipyrone in mice for 5 weeks. As shown in Table 1, the parameter relating to lipid metabolism (TG, total cholesterol and non-esterified fatty acid) in the HFD+KAL group did not show significant differences against those of the HFD group. On the other hand, the Glc level (155.1 ± 28.2 mg dl^{-1}) was relatively higher than that of the HFD group (135.7 ± 16.6 mg dl^{-1}), and the plasma LA level (95.4 ± 2.4 mg dl^{-1}) in the HFD+KAL group was significantly increased ($P<0.05$) when compared with that of the HFD group (68.8 ± 6.1 mg dl^{-1}). These characteristic changes in plasma LA levels and adipose tissue are supported by the results of the *in vitro* experiments with yoshinone A, as described above. Incidentally, the enhancement of LA production will have the potential for causing the physiological problem by lactic acidosis, but our autopsy studies and behavior observations did not find any abnormal changing and health hazard in the kalkipyrone-treated mice during experiment for 5 weeks. In the near future, further studies including physiological pH monitoring will elucidate that there are no adverse impact by elevated blood lactic level induced by these γ-pyrone treatment in mice.

To summarize, anti-obesity activities of the 7-en γ-pyrone have been shown in experiments in both cultivated cells and in mice. In the mature 3T3-L1 adipocytes, the reducing effects of yoshinone A on accumulated TG amounts accompanied with the enhancement of LA production in the culture fluid were observed *in vitro*. In the HFD feeding mice, the suppressive effects of orally ingested kalkipyrone on adipose tissue weight gain for 5 weeks was accompanied with an enhancement of plasma LA level *in vivo*. On the basis of these preliminary results, it was suggested that the 7-en γ-pyrone expresses an anti-obesity effect *in vivo* with oral administration, and the enhancement of LA production will be a key phenomenon in the reduction of accumulated TG in adipocytes. LA is a major end product of Glc metabolism by the glycolytic system in the cytosol, but, as usual, the citric acid cycle in the mitochondria suppresses LA production by the consumption of Glc metabolites to produce energy. Ubiquinone distributing in the mitochondrial inner membrane has an important role in the mitochondrial respiratory chain, mediating electron transport between NADH and succinate dehydrogenase, and the cytochrome system.[13] The molecule of ubiquinone contains unconjugated olefin with a quinone ring similar to the 7-en γ-pyrone, such as yoshinone A. We speculate that this unconjugated type of pyrone acts as a mimic of ubiquinone in the mitochondrial membrane to suppress functions, including the citric acid cycle. As a result, yoshinone A induced the accumulation of LA produced as a metabolite from the glycolytic system, and promoted fat utilization by compensating for the deficient energy supplying *in vitro* and *in vivo*. These speculations about bioactivities of the 7-en γ-pyrone have been not yet established due to a lack of supply of these compounds from natural sources. We are currently synthesizing the yoshinopyrones to determine their stereochemistries and to elucidate their detailed activity via further experiments *in vitro* and *in vivo*.

EXPERIMENTAL PROCEDURE

In vitro experiments in 3T3-L1 adipocyte

The reducing effect of yoshinone A on accumulated TG in adipocytes was evaluated using 3T3-L1 cells after differentiation. The murine preadipiocyte 3T3-L1 cells (Riken BRC, Tsukuba, Japan) were cultured in Dulbecco's modified Eagle's medium (Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco) in two 96-well plates at 37 °C, 5% CO_2. Two days after confluence, the differentiation was induced by Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 0.5 mM of 3-isobutyl-1-methylxanthine, 0.25 μM of dexamethasone each from Wako Pure Chemical Industries, Ltd. (Osaka, Japan)) and 10 μg ml^{-1} insulin (Gibco) for 7 days.

The differentiated 3T3-L1 adipocytes in a 96-well plate were treated with 2% Triton-X 100 for 30 min at room temperature followed by sonication. Accumulated TG amounts and cell viability were evaluated referring to the method with by Inuzuka *et al.*[5]

Anti-obesity test in mice

Male ddY mice were obtained from Japan SLC, Inc. (Shizuoka, Japan). They were housed in individual cages under a 12-h/12-h light/dark cycle (lights from 0800 to 2000 hours) in a room with controlled temperature and humidity (25 ± 1 °C and 60 ± 5%, respectively). For the experiment, we formulated experimental diets based on the AIN-93M diet.[14] To mimic a westernized diet rich in animal fat, we used HFD-60 (Oriental Yeast Co., Ltd., Tokyo, Japan) including lard as fat (62.2 kcal%) in the experimental HFDs. Male mice (5 weeks old) were fed a ND for 1 week and then divided into the following three groups: ND ($n=6$); HFD ($n=6$); and HFD+KAL ($n=3$). Kalkipyrone (5 mg kg^{-1} per day) was administered orally to the mice fed a HFD (HFD +KAL group). Other mice received vehicle (10 ml kg^{-1} per day) orally. Body weight, food intake and drinking water were measured every days. After the mice were fed these diets for 5 weeks. The feces were collected for the last 3 days and dried to weigh. The mice were killed by anesthetic overdose with isoflurane. And then, blood was collected from the abdominal vein to prepare plasma, and the epididymal adipose tissue and liver were dissected and weighed. The TG in the liver and feces were extracted with methanol–chloroform solution following homogenization. The plasma TG, total cholesterol, non-esterified fatty acid, Glc and LA levels were measured using the commercially clinical assay kit (Wako Pure Chemical Industries, Ltd.) for each. Data were presented as mean ± s.e. and analyzed by one-way analysis of variance and the Dunnett's test. Differences between groups were considered to be statistically significant at $P<0.05$.

Animal studies were performed in accordance with notification number 88 of the Ministry of the Environment, Japan, (2006) and the Guidelines for Animal Experimentation of the Tokyo University of Marine Science and Technology, with the approval of the Animal Care and Use Committee of the Tokyo University of Marine Science and Technology.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)