Malabaricone C as natural sphingomyelin synthase inhibitor against diet-induced obesity and its lipid metabolism in mice

Muhamad Aqmal Othman,[†] Kohei Yuyama,[‡] Yuta Murai,[§] Yasuyuki Igarashi,[‡] Daisuke Mikami,[‡] Yasodha Sivasothy,[⊥] Khalijah Awang,[‖] and Kenji Monde*[,§]

[†] Graduate School of Life Science, Hokkaido University, Kita 21 Nishi 11, Sapporo 001-0021, Japan

[‡] Lipid Biofunction Section, Faculty of Advanced Life Science, Hokkaido University, Kita 21 Nishi 11, Sapporo, 001-0021, Japan.

[§] Faculty of Advanced Life Science, Hokkaido University, Kita 21 Nishi 11, Sapporo 001-0021, Japan

[⊥] Research Centre for Crystalline Materials, Faculty of Science and Technology, Sunway University, 47500 Bandar Sunway, Selangor Darul Ehsan, Malaysia.

[‖] Department of Chemistry, Faculty of Science, University of Malaya, 50603, Kuala Lumpur, Malaysia

Supporting Information

1-Experimental procedures

General experimental method

All chemicals and solvents were obtained from commercial supplier and used without further purification. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were recorded on a Varian Inova instrument at 25 °C in $CDCl_3$ and CD_3OD purchased from Cambridge Isotope Laboratories, Inc. (Tewksbury, USA). Chemical shifts (δ) are reported in ppm and coupling constant values (*J*) are in hertz relative to $CDCl_3$ (^{1}H, δ 7.26; ^{13}C, δ 77.00) or CD_3OD (^{1}H, δ 3.4, 4.8; ^{13}C, δ 49.3) and tetramethylsilane. The following abbreviations were used for signal multiplicities: s = singlet; d = doublet; t = triplet; q = quartet; p = pentet and m = multiplet. High Resolution Mass spectra (HRMS (ESI)) were obtained from AB sciex Triple TOF® 5600+ at Platform for Research on Biofunctional Molecules, Hokkaido University. Analytical TLC was performed on 0.2 mm silica gel plates (Merck 60 F-254). SiO_2 gel column chromatography was carried out using silica gel (Wakogel® N60, spherical, 38-100 μm) with air flashing. The purity of isolated compounds was determined by NMR, all the compounds have purity of >90-95%.

Plants material

Myristica cinnamomea King was collected from Johor, Malaysia in 2003. The plant was identified by Mr. Teo Leong Eng and a voucher specimen (KL 5043) was deposited at the University of Malaya herbarium.

Isolation of active compounds from *M. Cinnamomea*

Dried and powdered fruits (1.5 kg) of *M. cinnamomea* was extracted with ethyl acetate (5.0 L), at room temperature two times, yielding 263 g of a dark brown extract and checked for SMS assay. The active ethyl acetate extract (35 g) had higher inhibition activity against SMS and bioassay guided fractionation of the *M. cinnamomea* resulted in the isolation of malabaricones

A-C (**1-3**) and malabaricone E (**4**). All isolated known compounds were confirmed by NMR spectroscopy, HRMS and having similar spectroscopic data with those reported in the literatures.[3,4] The yield obtained are as followed: Malabaricones A-C (5.58 g, 2.92 g, 6.04 g) and Malabaricone E (7.10 mg).

Malabaricone A (**1**) was obtained as a pale yellow, amorphous powder; HRMS (*m/z*): $[M + H]^+$, calculated for $C_{21}H_{27}O_3$: 327.1954; found : 327.1950. ^{1}H NMR (MeOH-d_4, 500 MHz): δ = 7.21 (2H, m), 7.18 (1H, t, J = 8.2 Hz), 7.15 (3H, m), 6.34 (2H, d, J = 8.2 Hz), 3.10 (2H, t, J = 7.3 Hz), 2.58 (2H, t, J = 7.3 Hz), 1.66 (2H, p, J = 7.3 Hz), 1.59 (2H, p, J = 7.3 Hz), 1.33 (8H, br s). ^{13}C NMR (MeOH-d_4, 125 MHz): δ = 209.8, 163.6, 163.6, 144.1, 137.0, 129.5, 129.5, 129.4, 129.4, 126.7, 111.5, 108.5, 108.5, 45.9, 37.1, 32.9, 30.8, 30.7, 30.6, 30.4, 25.9.

Malabaricone B (**2**) was obtained as a pale yellow, amorphous powder; HRMS (*m/z*): $[M + H]^+$, calculated for $C_{21}H_{27}O_4$: 343.1903; found : 343.1903. ^{1}H NMR (MeOH-d_4, 500 MHz): δ = 7.19 (1H, d, J =8.2 Hz), 6.97 (2H, d, J = 8.2 Hz), 6.67 (2H, d, J = 8.2 Hz), 6.33 (2H, d, J = 8.2 Hz), 3.11 (2H, t, J = 7.3 Hz), 2.49 (2H, t, J = 7.3 Hz), 1.67 (2H, p, J = 7.3 Hz), 1.55 (2H, p, J = 7.3 Hz), 1.34 (8H, br s). ^{13}C NMR (MeOH-d_4, 125 MHz): δ = 209.8, 163.6, 163.6, 144.1, 137.0, 129.5, 129.5, 129.4, 129.4, 126.7, 111.5, 108.5, 108.5, 45.9, 37.1, 32.9, 30.8, 30.7, 30.6, 30.4, 25.9.

Malabaricone C (**3**) was obtained as a yellow, amorphous powder; HRMS (*m/z*): $[M + H]^+$, calculated for $C_{21}H_{27}O_5$: 359.1853; found : 359.1862. ^{1}H NMR (MeOH-d_4, 500 MHz): δ = 7.19 (1H, d, J = 8.2 Hz), 6.65 (1H, d, J = 8.2 Hz), 6.60 (1H, d, J = 2.0 Hz), 6.47 (1H, dd, J = 8.2, 2.0 Hz), 6.34 (2H, d, J = 8.2 Hz), 3.11 (2H, t, J = 7.3 Hz), 2.44 (2H, t, J = 7.3 Hz), 1.67 (2H, p, J = 7.3 Hz), 1.54 (2H, p, J = 7.3 Hz), 1.33 (8H, br s). ^{13}C NMR (MeOH-d_4, 125 MHz): δ = 209.8, 163.6, 163.6, 146.1, 144.2, 137.0, 135.9, 120.8, 116.6, 116.3, 111.5, 108.5, 108.5, 45.9, 36.4, 33.1, 30.8, 30.7, 30.7, 30.4, 25.9.

Malabaricone E (**4**) was obtained as a yellow, amorphous powder; HRMS (*m/z*): $[M + H]^+$, calculated for $C_{21}H_{27}O_5$: 359.1853; found : 359.1861. ^{1}H NMR (MeOH-d_4, 400 MHz): δ = 6.97 (2H, d, J = 8.0 Hz), 6.67 (2H, d, J = 8.0 Hz), 5.80 (2H, s), 3.02 (2H, t, J = 8.0 Hz), 2.49 (2H, t, J = 8.0 Hz), 1.65 (2H, p, J = 8.0 Hz), 1.56 (2H, br t, J = 8.0 Hz), 1.33 (8H, br s). ^{13}C NMR (MeOH-d_4, 100 MHz): δ = 206.1, 164.6, 164.4, 164.4, 154.8, 133.5, 128.8, 128.8, 114.5, 114.5, 103.9, 94.3, 94.3, 43.4, 34.6, 31.6, 29.2, 29.2, 29.1, 28.9, 24.8.

Cell culture

The mouse embryonic fibroblast (tMEF) containing ZS/SMS1 and ZS/SMS2, WT-tMEF were presented from Prof. Igarashi (Lipid Bio Section, Faculty of Advanced Life Science, Hokkaido University). ZS cells were isolated from an SMS1, SMS2 double KO MEF, which were immortalized using SV40T antigen. ZS cells stably expressed SMS1 or SMS2, were named ZS/SMS1 and ZS/SMS2.[1] HepG2 cells were obtained from ATCC. Cells were cultured in dulbecco's modified eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) penicillin with streptomycin at 34 °C in a 5% CO_2 incubator.

Measurement of SMSs inhibitory activity in cell lysates

Cell lysates were prepared as follows: ZS/SMS1 and ZS/SMS2 cells (protein concentration 0.1 μg/μL) were diluted by 20 mM Tris-buffer (pH 7.5) and sonicated. Aliquots of the cell lysates 100 μL were added with 1 μL of inhibitor of desired concentration and incubated at 37°C. After 30 min, the solutions were added with 1 μL of C6-NBD-ceramide (5 μM), and incubated for 3 h at 37°C. The reaction was stopped by addition of 400 μL of Methanol/Chloroform [1/2 (v/v)]; the mixture was shaken and centrifuged (1500 rpm x 5 min). The formation of C6-NBD-sphingomyelin was quantified by determination of the peak area of C6-NBD-sphingomyelin using high performance liquid chromatography (HPLC). A reverse phase HPLC assay using a JACSO HPLC system was developed for the quantitative analysis of the inhibitory activity.

The system was equipped with a PU-2089 Plus and FP-2020 Plus set at λex = 470 nm and λem = 530 nm. A 50 x 4.6 YMC-Pack Diol-120-NP column (5-µm particle size) was used with mobile phase (IPA/hexane/water) at a flow rate of 1.0 mL/min.[2]

Measurement of SMSs inhibitory activity in cells

The tMEF cells were seeded at a density of 3.0×10^5 in 10 ml of DMEM medium containing 10% (v/v) fetal bovine serum (FBS), 1% (v/v) penicillin with streptomycin per 96 well-plate and cultured at 34 °C overnight. The medium was removed from the well-plate and 100 µl of inhibitor in medium containing 1% ethanol was added and incubated at 37 °C for 1 hour. The reaction then proceeds with an addition of 1 µl of C_6-NBD-ceramide (400 µM) in medium solution and incubated at 37 °C for 1 hour. The reaction mixture was removed and washed with 100 µl of PBS. The cell was digested with 20 µl of trypsin and incubated at 37 °C for 1 minute. Before transferring the whole amount of cell mixture to a deep well plate, 80 µl of PBS was added. The reaction was stopped and analyse in similar manner as previously described in cell lysate assays.

Animal care and experiments

All procedures were performed in accordance with guidelines by the animal research committee of Hokkaido University. Male C57BL/6 mice (five-week-old, Japan SLC Inc. Shizuoka, Japan) were housed in a temperature and humidity controlled cage (24 °C, 50% ± 10% relative humidity) with a 12 h light : dark cycle. The mice were sorted into 4 groups (n = 7-8 each) based on their body weight. The groups are as follows: 1) normal diet; vehicle control group (ND, 6.2% calories from fat, AIN-93M, Oriental Yeast Co., ltd, Japan), 2) high-fat diet; vehicle control group (HFD, 60% calories from fat, HFD-60, Oriental Yeast Co., ltd, Japan), and 3) HFD + 0.1% Compound 3 diet; treatment group. The mice were given free access to food ad libitum and water, meanwhile the weight change and feed intake were measured

weekly. The animal experiments continued for 9 weeks. Body weight, blood glucose and liver function were recorded.

Tissue sampling

After treatment, animals were anaesthetized with ketamine and xylazine, blood was sampled from the hearts and perfused intracardially with 40 ml of saline (0.9% NaCI). Liver tissues were precisely dissected, weighed and snap-frozen in liquid nitrogen and stored at −80 °C until further analysis.

Histopathological examination

Sample of liver for each group were resected and fixed with 4% formaldehyde phosphate buffered with saline pH 7.4, then embedded in paraffin, sectioned, stained with Oil Red O staining and the images were captured by Axio Imager M2 (Zeiss).

Oral glucose tolerance test (OGTT)

Oral glucose tolerance test was performed by oral administration of glucose solution (1 mg/g) after overnight fasting. The level of blood glucose was measured using a glucose meter (Accu Check, Roche, Switzerland) before oral glucose load (0 min); 30, 60 and 120 minutes after oral glucose load. The data collected during OGTT was plotted and calculated by GraphPad Prism.

Biochemical analysis

Levels of blood and hepatic triglycerides (TG) and free fatty acids (FFA) were measured as previously described by using TG and FFA enzyme-linked immunosorbent assay (ELISA) kit from (Wako, Japan) following the manufacturer's instructions. The bioassay was conducted using a 96-well plate, and the absorbance was determined at 550 nm for FFA and 595 nm for TG using a microplate reader (Model 680, BIO-RAD, Japan).

Measurement of DAG and SM levels in liver tissues lysates

Liver samples were weighted, homogenized, and centrifuged to get supernatant as lysates solution. The reaction and analysis were similar as previously described in cell lysate assays. The DAG and SM levels were calculated by GraphPad.

Measurement of intracellular triglycerides and free fatty acid levels.

1 X 10^5 cells/well of HepG2 cells were seeded in a 12-well plate and incubated at 37 ℃ in 5% CO_2. The next day, the cells were treated with 1 mM of oleic acid in FBS-free medium. Cells were incubated for 1 day in a CO_2 incubator at 37 ℃. Cells were washed with PBS and scraped to measure the triglycerides (TG) and free fatty acids (FFA) levels as previously described.

Immunofluorescence of lipid droplets from the oleic acid uptake.

1 X 10^5 cells/well of HepG2 cells were seeded in a 12-well plate and incubated at 37 ℃ in 5% CO_2. The next day, the cells were treated with 1 mM of oleic acid in FBS-free medium. Cells were incubated for 1 day in a CO_2 incubator at 37 ℃. After washing with PBS, the cells were fixed with 4% of paraformaldehyde in PBS for 30 minutes. Subsequently, cells were washed with PBS and stained with 2.5 mg/ml of Nile red solution for 15 minutes. Next, cells were stained with DAPI solution for 2 minutes and continue washed with PBS. The images were captured using a fluorescence microscope BZ-X70 (Keyence, Osaka, Japan) and analysed using ImageJ.

2-Analysis data

Table S1 show mode of inhibition analysis of compounds **1**-**3**.

Compounds	IC_{50}, µM			
	NBD-Ceramide, 5 µM		NBD-Ceramide, 50 µM	
	SMS 1	SMS 2	SMS 1	SMS 2
1	3	3	3	3
2	2	2	2	2.5
3	2.5	1.5	2	1

Changes in substrate concentration do not significantly affect the IC_{50} value of compounds **1**-**3** suggesting that compounds **1**-**3** is a non-competitive inhibitor of SMS 1 & 2.

Figure S1 show the cytotoxicity of compound **3** using cell counting kit-8 assay.

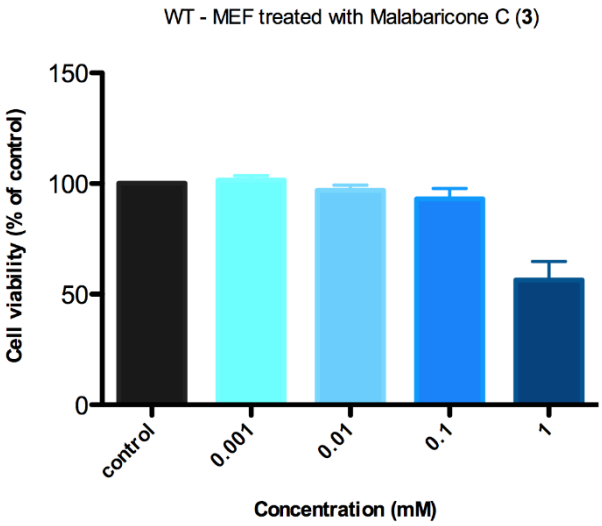

Figure S1. Cell counting kit-8 assay was used to evaluate the cytotoxic activity of compound **3** against wild-type mouse embryonic fibroblasts cells, MEF. Cell viability were analysed after 3 hours of treatment with compound **3** at concentration levels of 1-0.001 mM. Data are presented as the mean ± standard error of the mean (SEM)

Table S2 show the inhibitory activity of Sphingomyelin synthase (cell assay).

Compound	(IC_{50}, µM)	
	SMS 1	SMS 2
3	13	11

IC_{50} values are the means of three separate determinations on SMS1 or SMS2 expressed SMS1/2 double knockout mouse fibroblast cell lysate and were determined by more than four concentrations of each inhibitor.

Figure S2 show the in vivo result for daily food intake.

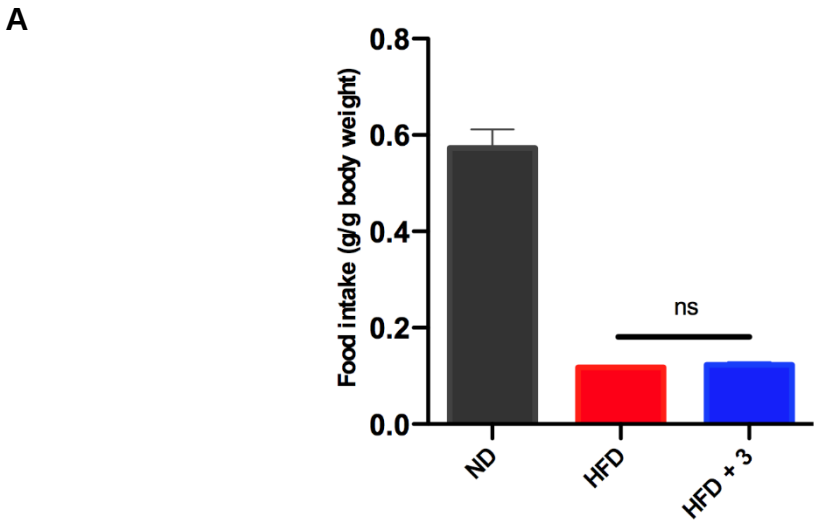

Figure S2. In vivo results of compound **3** on food intake. Control: ND, normal chow diet and HFD, High fat diet; Test group: HFD + **3**, High fat diet with 0.1% of Malabaricone C. Data are presented as mean ± standard error of the mean (SEM); *N* = 7-8 mice per group. Statistical analysis was done by using t-test: (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$, (****) $P < 0.0005$, ns = no significant difference versus the control.

3-References

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