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ARTICLE

Helvamide, a new inhibitor of sterol *O*-acyltransferase produced by the fungus *Aspergillus nidulans* BF-0142

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Abstract

A new piperazine derivative designated helvamide was isolated as a pair of rotamers (**1** and **2**) from the culture broth of the fungus *Aspergillus nidulans* BF-0142 along with a known helvafuranone (**3**). The structures of **1** and **2** were elucidated based on spectroscopic analyses by the interpretation of one-dimensional and two-dimensional nuclear magnetic resonance data, ROESY (rotational Overhauser effect spectroscopy) correlations, and a chemical method. Helvamide existed as a rotameric mixture (**1** and **2**) in dimethyl sulfoxide. Helvamide inhibited sterol *O*-acyltransferases 1 and 2 (SOAT1 and SOAT2) in enzyme-based and cell-based assays using SOAT1-expressing and SOAT2-expressing Chinese hamster ovary (CHO) cells.

Introduction

The challenge to discover new biologically active compounds from natural resources, such as animals, plants, and microorganisms, is continuing. Of these, microorganisms are expected to be the most important resource for new drug discovery [1]. A large number of studies that mainly focused on terrestrial microorganisms as drug discovery resources demonstrated that they have the potential to produce biologically active compounds, namely, antibiotics, anticancer, immunosuppressive, and dyslipidemic agents [2, 3]. We have recently been interested in poorly studied resources including marine-derived and hot spring-derived microorganisms. In the course of our investigations using these microorganisms, we identified the following novel compounds: seriniquinone [4] (an anticancer agent against melanoma cells), isomethoxyneihumicin [5] (cell cycle inhibitor), and graphiumins [6, 7] (inhibitors of yellow pigment production in methicillin-resistant *Staphylococcus aureus*). In our previous study, the *Aspergillus nidulans* BF-0142 strain, also isolated from soil collected at the hot spring area of Jigokudani in Noboribetsu, Hokkaido, Japan, was found to produce a new furanone metabolite designated helvafuranone (**3**) [8] along with five known compounds from broth cultured in Yunohana (concentrates of hot spring water)-containing medium. In continuing investigations, a new metabolite designated helvamide was isolated from the same culture broth as an inhibitor of sterol *O*-acyltransferase (SOAT), which catalyzes the synthesis of cholesteryl ester (CE) from free cholesterol and long-chain fatty acyl-coenzyme A (Fig. 1). Helvamide was found to be a mixture of rotamers **1** and **2**. In the present study, we described the fermentation, isolation, structural elucidation, and SOAT inhibitory activity of helvamide.

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Results

Collection and identification of strain BF-0142

Strain BF-0142 was isolated from soil collected at Jigokudani in Noboribetsu, Hokkaido, Japan in 2012 [8]. Based on

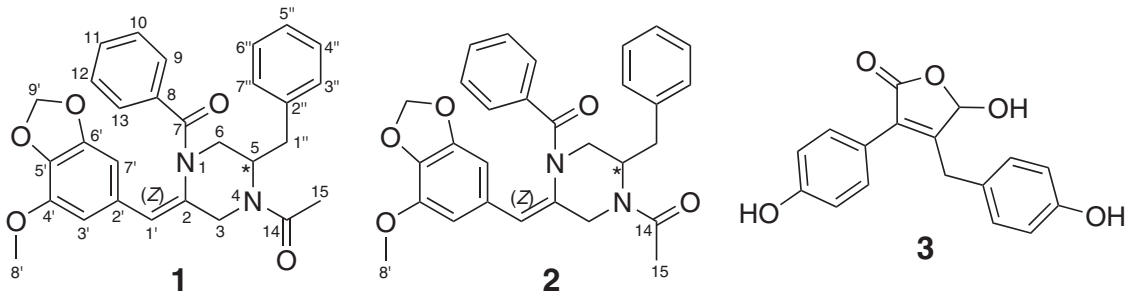

Fig. 1 Structures of helvamide (**1** and **2**) and helvafuranone (**3**)

a BLAST search and its microscopic features, the strain was identified as an *A. nidulans*.

Fermentation

The strain was inoculated into a 500-ml Erlenmeyer flask containing 100 ml seed medium (2.4% potato dextrose broth (Becton Dickinson, Sparks, MD, USA) and 0.1% agar (SSK sales, Shizuoka, Japan), pH 6.0). The flask was shaken on a rotary shaker at 27 °C for 4 days. The seed culture (1.0 ml) was transferred into a 1000-ml culture box containing 50 g production medium (50 g riso vialone nano rice (Masi Aglicola, Gargagnago, Italy), 1.2% potato dextrose broth, 1.0% malt extract (Becton Dickinson, Sparks, MD, USA), 1.0% glucose (Kanto Chemical Co., Inc., Tokyo, Japan), 0.05% peptone (Becton Dickinson, Sparks, MD, USA), and 0.04% yunohana (Tororon Kikaku, Hokkaido, Japan)). Fermentation was performed at 27 °C for 14 days under static conditions.

Isolation

A 14-day-old culture of the strain (50 g × 30) was extracted with 70% ethanol (6.0 l) for 2 h. Ethanol extracts were concentrated to remove ethanol, and the aqueous solution was extracted with ethyl acetate, yielding crude material (6.6 g) after the evaporation of ethyl acetate. The material was dissolved in a small volume of chloroform, applied to a silica gel column (350 g, $58 \times 450\ mm^2$), and eluted stepwise with 100% chloroform, 50:1, 25:1, 10:1, 5:1, and 1:1 (v v^{-1}) of chloroform-methanol, and 100% methanol (1000 ml each). A crude sample containing helvamide (1.1 g) was obtained in the fraction eluted with 5:1 chloroform-methanol. The crude sample was further purified by high-performance liquid chromatography (HPLC) using a reversed-phase C-18 column ($10 \times 250\ mm^2$; PEGASIL ODS SP100, Senshu Scientific Co., Tokyo, Japan) under the following conditions: solvent, 40 to 60% aq. acetonitrile with 0.05% trifluoroacetic acid, linear gradient in 60 min; flow rate, 3.0 ml min^{-1}; detection, ultraviolet (UV) at 210 nm. Helvamide was eluted as a peak with a retention time of 34 min. Helvamide was a mixture of rotamers (**1** and **2**), which were not possible to separate. This peak was collected and concentrated to yield 8.6 mg.

Table 1 Physicochemical properties of helvamide **1** and **2**)

	1 and **2**
Appearance	Fluorescent yellow powder
Molecular formula	$C_{29}H_{28}N_2O_5$
Molecular weight	484
HR ESI-MS *m/z*	$(M + Na)^+$
Calcd	507.1895 (for $C_{29}H_{28}N_2O_5Na$)
Found	507.1888
UV (MeOH)	258
IR $\nu_{max}^{KBr} cm^{-1}$	1644, 1510

Physicochemical properties of helvamide (1 and 2)

The physicochemical properties of helvamide (a mixture of **1** and **2**) are summarized in Table 1. Helvamide was obtained as a fluorescent yellow powder. The molecular formula for helvamide was established as $C_{29}H_{28}N_2O_5$ ($[M + Na]^+$ *m/z* 507.1888, calcd $[M + Na]^+$ 507.1895) based on high-resolution electrospray ionisation-mass spectrometry (ESI-MS) measurements, indicating that helvamide contained 16 degrees of unsaturation. It showed an absorption maximum at 258 nm in the UV spectrum. Absorption at 1644 and 1510 cm^{-1} in the infrared (IR) spectrum suggested the presence of carbonyl and phenyl groups.

Structural elucidation of helvamide (1)

Helvamide existed as a mixture of **1** and **2** in an equilibrium of 3:1 in dimethyl sulfoxide-d_6 (DMSO-d_6) from nuclear magnetic resonance (NMR) data. Therefore, the structural elucidation of helvamide was performed as the mixture. ^1H and ^{13}C NMR data (in DMSO-d_6) were very complex; however, each signal was carefully assigned based on the signal intensity.

Table 2 NMR spectroscopic data for helvamide (**1** and **2**) in DMSO-d_6

	1			**2**	
Position	δ_C[a]	δ_H[b] mult (*J* in Hz)	HMBC	δ_C[a]	δ_H[b] mult (*J* in Hz)
2	133.5, C			133.3, C	
3	41.9, CH_2	4.07, d (13.5)	2, 14, 1′	47.2, CH_2	4.34, d (13.0)
		4.88, d (13.5)	2, 5, 14, 1′		4.43, d (13.0)
5	55.3, CH	4.29, brt (10.0)	3, 6, 14, 1″, 2″	49.7, CH	4.86, m[c]
6	46.7, CH_2	3.30[c],	2, 5, 7, 1″	44.6, CH_2	3.06, d (8.5)
		4.48, d (13.0)	2, 5, 7, 1', 1″		4.35, d (12.0)
7	169.2, C			169.1, C	
8	134.69, C			134.65, C	
9/13	127.1[d], CH	7.11, d (7.0)	7	127.0[d], CH	7.06, d (7.0)
10/12	127.1[d], CH	7.16[d], m	8	127.0[d], CH	7.16[d], m
11	129.94, CH	7.29[d], m		129.97, CH	7.30[d], m
14	168.3, C			168.5, C	
15	20.8, CH_3	1.66, s	5, 14	21.6, CH_3	2.04, s
1′	122.2, CH	6.01, s	2, 3, 2′, 3′, 7′	122.4, CH	6.15, s
2′	128.3, C			128.3, C	
3′	107.4, CH	5.92, d (1.0)	1′, 2′, 4′, 5′, 7′	107.3, CH	5.94, s
4′	143.0, C			143.1, C	
5′	133.9, C			134.0, C	
6′	148.3, C			148.4, C	
7′	101.43, CH	5.86, d (1.0)	1′, 3′, 5′, 6′	101.46, CH	5.84, s
8′	55.8, CH_3	3.64, s	4′	55.9, CH_3	3.64, s
9′	101.13, CH_2	5.91, d (1.0)	5′ 6′	101.16, CH_2	5.91, d (1.0)
		5.95, d (1.0)	5′ 6′		5.95, d (1.0)
1″	35.2, CH_2	2.97, dd (13.0, 6.0)	5, 6, 2″, 3″, 7″	34.8, CH_2	2.85, dd (13.0, 8.0)
		3.17, dd (13.0, 9.0)	5, 6, 2″, 3″, 7″		3.02, dd (13.0, 9.0)
2″	138.1, C			138.1, C	
3″/7″	129.5, CH	7.30[d], m	1″	129.2, CH	7.19[d], m
4″/6″	126.5, CH	7.24[d], m	2″	126.3, CH	7.26[d], m
5″	128.3, CH	7.30[d], m		128.3, CH	7.30[d], m

Assignments made by the interpretation of COSY, HSQC, and HMBC NMR data

[a]100 MHz

[b]400 MHz

[c]Overlapping with the HOD signal (3.35 ppm)

[d]Overlapping

The ^{13}C NMR spectrum of **1** showed 29 resolved signals, which were classified into 2 methyl signals, 4 methylene signals, 14 methine signals including 13 sp^2 methine signals, and 9 signals from carbons bearing no hydrogens, including two carbonyl carbon signals. The ^1H-NMR spectrum of **1** showed two singlet methyl signals including 1 oxygenated methyl signal, 4 methylene signals, 2 methine signals, and 12 aromatic proton signals. The connectivity of all protons and carbon atoms was established by heteronuclear multiple-quantum correlation experiments (Table 2). An analysis of ^1H-^1H correlated spectroscopy (COSY) data revealed two mono-substituted benzene rings and the linkage of C-6 (δ 46.7)-C-5 (δ 55.3)-C-1″ (δ 35.2) (Fig. 2a). An analysis of heteronuclear multiple bond correlation (HMBC) spectroscopic data provided further structural information on **1**. The cross-peaks from H_2-3 (δ 4.07, 4.88) to C-2 (δ 133.5) and C-5, from H-5 (δ 4.29) to C-3 (δ 41.9), and from H_2-6 (δ 3.30, 4.48) to C-2 supported the presence of the central piperazine ring. Furthermore, the cross-peaks from H-1′ (δ 6.01) to C-2′ (δ 128.3), C-3′ (δ 107.4), and C-7′ (δ 101.43), from H-3′ (δ 5.92) to C-1′ (δ 122.2), C-2′, C-4′ (δ 143.0), C-5′ (δ 133.9), and C-7′, from H-7′ (δ 5.86) to C-1′, C-3′, C-5′, and C-6′ (δ 148.3), from H-8′ (δ 3.64) to C-4′, and from H_2-9′ (δ 5.91, 5.95) to C-5′ and C-6′supported *O*-methoxy benzodioxole (partial structure I). Additional cross-peaks from H-3 to C-1′ and from

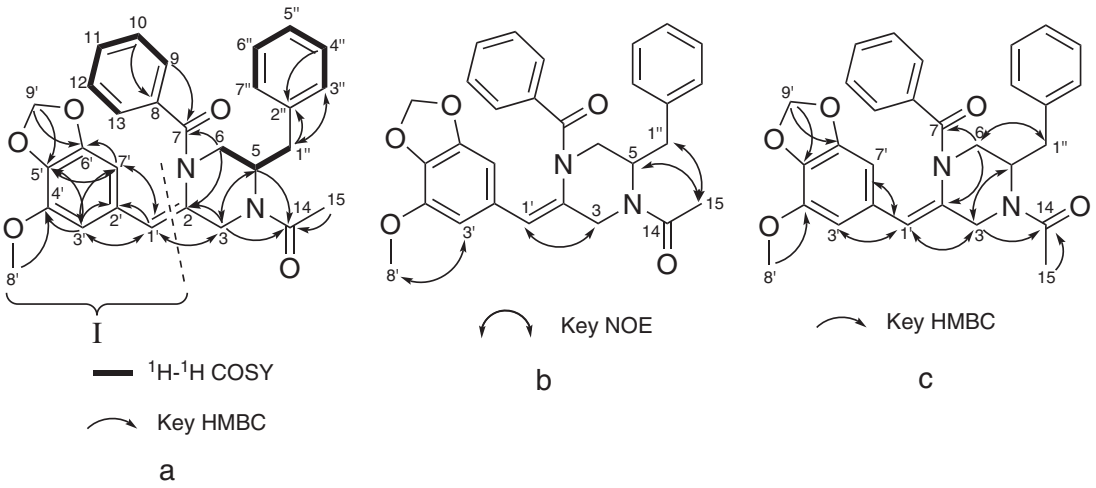

Fig. 2 2D NMR data of helvamide (**1** and **2**). **a** ^{1}H-^{1}H COSY and Key HMBC correlations of **1**. **b** Key NOESY correlations of **1**. **c** Key HMBC correlations of **2**

H-1′ to C-2 and C-3 supported the connection from the piperazine ring to partial structure I as shown in Fig. 2a. The cross-peaks from H_2-6 to C-7 (δ 169.2) and from aromatic H-9/13 (δ 7.11) to C-7 supported a benzoyl moiety being connected to the piperazine ring at N-1. The cross-peaks from H-3 and H-5 to C-14 (δ 168.3) and from H_3-15 (δ 1.66) to C-14 supported the presence of an *N*-acetyl moiety. The cross-peaks from H_2-1″ (δ 2.97, 3.17) to C-2" (δ 138.1) and C-3″/7″ (δ 129.5) and from aromatic H-3″/7″ (δ 7.30) to C-1″ supported a benzyl moiety being connected to the piperazine ring at C-5. Collectively, these data revealed the planar structure of **1** as shown in Fig. 2a. An unusual point of this structure is that the *N*-benzoyl aromatic protons (H-9, H-11, and H-13) in ^{1}H-NMR, generally observed at approximately δ 7.8, were shifted in higher fields (δ 7.11–7.29) in **1**. Based on molecular modeling, protons had a shield effect under the benzene ring of partial structure I. In order to prove the presence of an *N*-benzoyl moiety, helvamide was hydrolyzed with hydrochloric acid and the hydrolysate was analyzed using liquid chromatography-mass spectrometry, resulting in the identification of benzoic acid.

Stereochemistry of helvamide (1)

The conformation of the double bond between C-2 and C-1′ was elucidated by rotational Overhauser effect spectroscopy (ROESY) spectra (Fig. 2b). A nuclear Overhauser effect (NOE) correlation between H-3 and H-1′ was observed, supporting the *Z* configuration. Additionally, the observation of an NOE correlation between H-5 and H_3-15 supported the *N*-acetyl methyl and benzyl moieties being closely oriented.

Structural elucidation of helvamide (2)

In ^{1}H-NMR, the signals of **2**, such as H_3-15 (δ 2.04), were observed in a 1/3 integrated value of the counterpart signals of **1** (Table 2). A marked difference between **1** and **2** was observed in the chemical shifts of H_2-3, H-5, and H_3-15 in **2**. The signals of H_2-3 were observed at δ 4.34 and 4.43 instead of δ 4.07 and 4.88 in **1**. The signals of H-5 in **2** were shifted to a lower field (δ 4.86) than those of H-5 (δ 4.29) in **1**, suggesting that H-5 in **2** was affected by the C-14 carbonyl carbon. Fujita and Hayashi [9] reported brasiliamides with an *N*-acetyl piperazine ring showing a conformational change due to the restricted rotation of an amide bond in solution. The case of helvamide well agreed to their study. In addition, an NOE correlation was not observed between H-5 and H_3-15. By taking this into consideration, **2** was the rotamer of **1** in the direction of the *N*-acetyl moiety (Fig. 2).

Biological properties

Inhibition of CE synthesis in SOAT1-CHO and SOAT2-CHO cells

The effects of helvamide on the SOAT1 and SOAT2 isozymes were evaluated in a cell-based assay using SOAT1-expressing and SOAT2-expressing Chinese hamster ovary (CHO) cells. As shown in Table 3, helvamide inhibited CE synthesis in SOAT1-CHO and SOAT2-CHO cells with half-maximal inhibitory concentration (IC_{50}) values of 22 and 6.7 μM, respectively. Helvamide had no effects on phospholipid synthesis nor cytotoxic effects even at the highest dose of 44 μM in SOAT1-CHO and SOAT2-CHO cells.

Table 3 Effect of helvamide on SOAT1 and SOAT2 activities in cell-based and enzyme-based assays

	IC_{50} (μM) for CE synthesis					
	Cell-based assay[a]			Enzyme-based assay[a]		
	SOAT1	SOAT2	SI[b]	SOAT1	SOAT2	SI[b]
Helvamide	22	6.7	0.51	5.5	5.5	0.0

[a] $n = 3$

[b] SI (selectivity index) = log (IC_{50} for SOAT1)/(IC_{50} for SOAT2)

In order to confirm the inhibitory activity of helvamide against SOAT isozymes, enzymatic assays for SOAT1 and SOAT2 were performed using microsomal fractions prepared from the respective cells. As shown in Table 3, helvamide inhibited SOAT1 and SOAT2 activities with the same IC_{50} value of 5.5 μM. These results are consistent with those obtained in a cell-based assay.

Discussion

Helvamide (**1** and **2**) having *N*-benzoyl and *N*-acetyl moieties was isolated as a mixture of rotamers, which has not yet been separated. The rotamers **1** and **2** existed in a ratio of approximately 3:1, the *N*-acetyl moiety of which was oriented in a different direction (Fig. 1). The structure including its stereochemistry was elucidated, except for at the C-5 position. In order to elucidate the absolute configuration of C-5, circular dichroism (CD) was measured in methanol. Helvamide exhibited a strong negative Cotton effect at 310 nm and positive Cotton effect at 270 nm (see the Supporting Information). Shin et al. [10] reported that (2*Z*, 5*R*)-piperazinedione showed a negative Cotton effect at approximately 300 nm, while (2*Z*, 5*S*)-piperazinedione showed a positive Cotton effect [10]. If this role is applied to the piperazine case, the configuration is estimated to be 5*R*. In order to clarify this point, a total synthesis study is now ongoing.

The biological activity of helvamide was examined in in-house assays. The results obtained showed that helvamide inhibited SOAT1 and SOAT2 in cell-based and enzyme-based assays [11]. Structurally similar SOAT inhibitors were reported: EAB-309 and EAB-310 [12] and piperine [13] with a benzodioxole moiety and YIC-C8-434 [14] with a piperazine ring. Thus, helvamide is the first inhibitor to have benzodioxole and a piperazine ring in its structure.

General experimental procedures

ESI-MS spectrometry was conducted on an AccuTOF LC-plus T100LP mass spectrometer (JEOL, Tokyo, Japan). UV and IR spectra were measured with a U-2800 spectrophotometer (Hitachi, Tokyo, Japan) and FT/IR-460 plus spectrometer (JASCO, Tokyo, Japan), respectively. Optical rotations were obtained with a JASCO P-2200 polarimeter. CD spectra were measured on a JASCO J-700 spectropolarimeter. Various NMR spectra were measured with UNITY 400 (Agilent Technologies, Santa Clara, CA, USA). Reversed-phase HPLC separation was performed using a PEGASIL ODS SP100 column (10×250 mm^2) at a flow rate of 3.0 ml min^{-1} using the SHIMADZU LS20AT pump and SHIMADZU LS20AS UV detector. Absorbance was read with Power Wave 340 (Biotek Instruments Inc., Winooski, VT, USA).

Materials

[1-^{14}C]Oleic acid (1.85 GBq mmol^{-1}) and [1-^{14}C]oleoyl-CoA (1.85 GBq mmol^{-1}) were purchased from Perkin-Elmer (Waltham, MA, USA). Fetal bovine serum (FBS) was purchased from Biowest (Nuaille, France). Penicillin (10,000 U ml^{-1}) and streptomycin (10,000 mg ml^{-1}) solution were from Invitrogen (Carlsbad, CA, USA). Ham's F-12 medium was purchased from Nacalai Tesque (Kyoto, Japan). MEM vitamin solution and fatty acid-free bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Geneticin (G-418 sulfate) was purchased from Life Technologies Corporation (Carlsbad, CA, USA).

Cell culture

Two cell lines, CHO cells expressing the SOAT1 and SOAT2 isozymes of the African green monkey (SOAT1- and SOAT2-CHO cells, respectively) [15], were kind gifts from Dr. Lawrence L. Rudel (Wake Forest University, Winston-Salem, NC, USA) and were cultured using a previously described method [16].

Assays for SOAT activities in SOAT1-CHO and SOAT2-CHO cells

Assays for SOAT1 and SOAT2 activities using SOAT1-CHO and SOAT2-CHO cells were performed by our established method [16]. Briefly, SOAT1-CHO or SOAT2-CHO cells (1.25×10^5 cells) were cultured in each well of a 48-well plastic microplate (Corning Co., Corning, NY, USA) in 250 μl of Ham's F-12 medium supplemented with 10% heat-inactivated FBS, MEM vitamins, penicillin (100 U ml^{-1}), streptomycin (100 U ml^{-1}), and geneticin (300 μg ml^{-1}) and were allowed to recover at 37 °C overnight in 5.0% CO_2. Assays were conducted with cells that were at least 80% confluent. Following overnight recovery, the test sample (2.5 μl methanol solution) and [1-^{14}C]oleic acid (5.0 μl 10% ethanol/phosphate-buffered saline (PBS), 1 nmol, 1.85 kBq) were added to each well of the culture.

After an incubation at 37 °C for 6 h in 5.0% CO_2, the cells in each well were washed twice with PBS and lysed by adding 0.25 ml of 10 mM Tris-HCl (pH 7.5) containing 0.10% (w v^{-1}) sodium dodecyl sulfate, and cellular lipids were extracted by the method of Bligh and Dyer [17]. After concentrating the organic solvent, total lipids were separated on a thin-layer chromatography (TLC) plate (silica gel F254, thickness of 0.5 mm, Merck) in a solvent system of *n*-hexane:diethyl ether:acetic acid at a ratio of 70:30:1 (v v^{-1} v^{-1}) and analyzed with a bioimaging analyzer (FLA-7000; Fujifilm, Tokyo, Japan) to measure the amount of [^{14}C]CE (Rf: 0.77). The IC_{50} value was defined as a drug concentration causing the 50% inhibition of biological activity.

Preparation of microsomes from SOAT1-CHO or SOAT2-CHO cells

An enzyme source from SOAT1-/SOAT2-CHO cells using a Potter-type homogenizer was prepared using our established method [18]. SOAT1-CHO or SOAT2-CHO cells (2×10^8 cells) were homogenized in 10 ml of cold buffered sucrose solution (pH 7.2, hereafter referred to as buffer A, 100 mM sucrose, 50 mM KCl, 40 mM KH_2PO_4, and 30 mM EDTA) including protease inhibitors cocktail (Complete Mini (Roche)) in the Potter-type homogenizer. The microsomal fraction was pelleted by centrifugation at 100,000 × *g* at 4 °C for 1 h, resuspended in the same buffer at a concentration of 5.0 mg protein ml^{-1}, and stored at −80 °C until used.

Assay for SOAT activities in microsomes from SOAT1-CHO and SOAT2-CHO cells

SOAT1 and SOAT2 activities were assessed using microsomes prepared as described above. Briefly, an assay mixture containing 500 µg fatty acid-free BSA in buffer A and [1-^{14}C]oleoyl-CoA (20 µM, 3.7 kBq) together with a test sample (added as 10 µl methanol solution), and the SOAT1 or SOAT2 microsomal fraction (150 or 10 µg of protein, respectively) in a total volume of 200 µl were incubated at 37 °C. The SOAT reaction was started by adding [1-^{14}C] oleoyl-CoA. After a 5-min incubation, the reaction was stopped by adding 1.2 ml of chloroform/methanol (2:1). The product [^{14}C]CE was extracted by the method of Bligh and Dyer [17]. After the organic solvent was removed by evaporation, lipids were separated on a TLC plate and the radioactivity of [^{14}C]CE was measured as described above. The IC_{50} value was defined as a drug concentration causing the 50% inhibition of biological activity.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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