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ORIGINAL ARTICLE

7-Chlorofolipastatin, an inhibitor of sterol *O*-acyltransferase, produced by marine-derived *Aspergillus ungui* NKH-007

Ryuji Uchida[1], Kento Nakajyo[1], Keisuke Kobayashi[1], Taichi Ohshiro[1], Takeshi Terahara[2], Chiaki Imada[2] and Hiroshi Tomoda[1]

A new depsidone, named 7-chlorofolipastatin, and five known structurally related depsidones were isolated from the culture broth of the marine-derived fungus *Aspergillus ungui* NKM-007 by solvent extraction and HPLC using an octadecylsilyl column. The structure of 7-chlorofolipastatin was elucidated by various spectroscopic data including 1D and 2D NMR spectroscopy. 7-Chlorofolipastatin inhibited sterol *O*-acyltransferase (SOAT) 1 and 2 isozymes in cell-based and enzyme assays using SOAT1- and SOAT2-expressing Chinese hamster ovary (CHO) cells.
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INTRODUCTION

The enzyme sterol *O*-acyltransferase (SOAT, also known as acyl-coenzyme A:cholesterol acyltransferase), an endoplasmic reticulum membrane protein, plays important roles in cholesterol regulation in humans.[1] SOAT may be a promising target for the development of new anti-atherosclerotic agents. Although pharmaceutical laboratories have identified a number of synthetic SOAT inhibitors, none have been successfully developed to date because of side effects or the lack/absence of efficacy in clinical trials.[2–5] Recent molecular biological studies revealed the existence of two SOAT isozymes, SOAT1 and SOAT2, with distinct functions.[6–9] SOAT1 is ubiquitously expressed in all tissues and cells, whereas SOAT2 is expressed predominantly in the liver (hepatocytes) and intestine.[10]

Our group has an extensive experience in discovering SOAT inhibitors with a microbial origin in an enzyme assay using rat liver microsomes.[11]. After confirming the presence of two SOAT isozymes, we reexamined SOAT inhibitors in cell-based and enzyme assays utilizing Chinese hamster ovary (CHO) cells expressing African green monkey SOAT1 (SOAT1-CHO cells) and SOAT2 (SOAT2-CHO cells) to investigate the selectivity of inhibitors toward these isozymes.[12,13] We have continued this screening program in consideration of the selectivity of candidate microbial cultures toward the SOAT isozymes in these assay systems. A new chlorinated depsidone, designated 7-chlorofolipastatin (**1**; Figure 1), was recently isolated along with five known depsidones: folipostatin (**2**),[14] unguinol (**3**),[15] 2-chlorounginol (**4**),[16] 2,7-dichlorounginol (**5**)[17] and nornidulin (**6**)[18] from the culture broth of the marine-derived fungus *Aspergillus ungui* NKH-007. The fermentation, isolation, structural elucidation and SOAT inhibitory activities of **1** to **6** have been described in the present study.

RESULTS AND DISCUSSION

Isolation of 1 to 6

The culture broth (0.6 l) was treated with EtOH (0.6 l) for 1 h, and EtOH extracts were filtered to remove cell debris. After the concentration of extracts to remove EtOH, the aqueous solution was adjusted to pH 9.0 and extracted with EtOAc (0.6 l). The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure to give brown materials (307 mg). These materials were dissolved in a small amount of MeOH and purified by HPLC under the following conditions: column, CAPCELL PAK C18 (i.d. 20 × 250 mm; Shiseido, Tokyo, Japan); mobile phase, 50% CH_3CN containing 0.05% H_3PO_4; flow rate, 8 ml min^{-1}; detection, UV 210 nm. Compounds **1** to **6** were eluted a peak with retention times of 78, 41, 22, 34, 55 and 83 min, respectively. Each fraction of the peak was collected and concentrated to remove CH_3CN. The aqueous solution was extracted with EtOAc and the organic layer was concentrated to dryness to give pure **1** (27.3 mg), **2** (19.3 mg), **3** (33.0 mg), **4** (26.0 mg), **5** (23.4 mg) and **6** (15.7 mg) as white powders.

Structural elucidation of 1

The physicochemical properties of **1** are summarized in Table 1. In UV spectra, **1** showed absorption maxima at 226 and 278 nm in MeOH. Broad OH absorption near 3414 cm^{-1}, typical C-H (CH_2) stretching absorptions at 2924 and 2857 cm^{-1}, carbonyl absorption at 1711 cm^{-1} and aromatic C-C stretch absorptions (for carbon–carbon

[1]Graduate School of Pharmaceutical Sciences, Kitasato University, Tokyo, Japan and [2]Graduate School of Marine Science and Technology, Tokyo University of Marine Science and Technology, Tokyo, Japan
Correspondence: Professor H Tomoda, Graduate School of Pharmaceutical Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan.
E-mail: tomodah@pharm.kitasato-u.ac.jp
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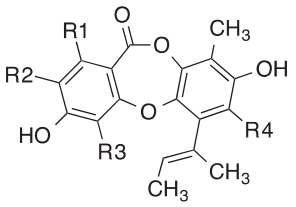


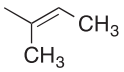
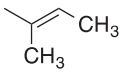
	R1	R2	R3	R4
7-Chlorofolipastatin (**1**)		–H	$-CH_3$	–Cl
Folipastatin (**2**)		–H	$-CH_3$	–H
Unguinol (**3**)	$-CH_3$	–H	$-CH_3$	–H
2-Chlorounguinol (**4**)	$-CH_3$	–Cl	$-CH_3$	–H
2,7-Dichlorounguinol (**5**)	$-CH_3$	–Cl	$-CH_3$	–Cl
Nornidulin (**6**)	$-CH_3$	–Cl	–Cl	–Cl

Figure 1 Structures of 7-chlorofolipastatin (**1**) and related compounds **2** to **6**.

Table 1 Physicochemical properties of 1

Appearance	White powder
Molecular formula	$C_{23}H_{23}O_5Cl$
Molecular weight	414
HR-ESI-MS (m/z)	
Calcd	437.1137[M+Na]$^+$
Found	437.1134[M+Na]$^+$
UV$\lambda_{max}^{CH_3OH}$ nm (log ε)	226(2.19), 278(1.11)
IRv $\frac{KBr}{max}$ cm^{-1}	3414, 2924, 2857, 1711, 1600, 1412, 1267, 1122, 1078, 822
$[\alpha]_D^{26}$(c = 0.1, CH_3OH)	0°
Solubility	
Soluble	$CHCl_3$, EtOAc, CH_3CN, CH_3OH
Insoluble	H_2O

Abbreviation: ESI, electrospray ionization.

bonds in the aromatic ring) at 1600 and 1412 cm^{-1} were observed in IR spectra. The electrospray ionization (EI)-MS of **1** showed an isotope pattern of *m/z* 413 [M-H]$^-$ and 415 [M+2-H]$^-$ at a ratio of 3:1, indicating the presence of one chlorine atom in the molecule. The molecular formula $C_{23}H_{23}O_5Cl$ was assigned on the basis of its EI-MS (*m/z* 437.1134 [M+Na]$^+$, Δ−0.3 mmu), indicating 12 degrees of unsaturation.

The ^{1}H and ^{13}C NMR spectra of **1** in DMSO-d_6 (Table 2) showed 23 proton and 23 carbon signals that were confirmed by an analysis of 2D NMR correlations. The multiplicity of the carbon signals was classified into five methyl carbons, three sp^2 methine carbons, one sp^2 nitrogenated methine carbon, eight sp^2 quaternary carbons, five sp^2 oxygenated quaternary carbons and one carbonyl carbon by an analysis of distorsionless enhancement by polarization transfer (DEPT) and heteronuclear multiple-quantum correlation (HMQC) data. The connectivity of proton and carbon atoms was established by HMQC (Table 2). As shown in Figure 2, the partial structures I and II were elucidated by ^{1}H–^{1}H correlation spectroscopy (COSY) spectra. The ^{13}C–^{1}H long-range couplings of ^2J and ^3J in the heteronuclear multiple bond correlation (HMBC) spectra (Figure 2) proved the presence of the following linkages. (1) Cross peaks from the sp^2 methine proton H-2 (δ 6.55) to the sp^2 quaternary carbon C-3 (δ 159.9), the sp^2 quaternary carbon C-4 (δ 113.3) and the sp^2 carbon C-11a (δ 110.0), from the hydroxyl proton OH-3 (δ 10.54) to C-3 and the sp^2 carbon C-4 (δ 113.3) and from the methyl proton 4-CH_3 (δ 2.04) to C-3, C-4 and the sp^2 oxygenated carbon C-4a (δ 161.3) suggested the presence of a 3-hydroxy-4-methyl-1,4a,11a-pentasubtituted benzene ring. Further long-range couplings from the sp^2 methine proton H-2′ (δ 5.34) to the methyl carbon C-4′(δ 17.5), from the methyl proton H_3-3′ (δ 1.64) to the sp^2 carbon C-1′ (δ 135.7) and from the methyl proton H_3-4′ (δ 1.77) to C-1′ and the sp^2 methine carbon C-2′ (δ 123.9) suggested the presence of an isobutyl group containing the partial structure I. This isobutyl group was connected to the benzene ring at C-1 by an observation of the ^3J cross peaks from H-2′ and H_3-4′ to the carbon C-1 (δ 148.4) and of the ^4J cross peak from H-3′to C-1 in HMBC experiments. Furthermore, a ^4J cross peak was observed from H-2 to the carbonyl carbon C-11 (δ 163.0) in HMBC experiments, suggesting that this carbonyl carbon was connected to the benzene ring at C-11a. Thus, the ring A of **1** was elucidated as shown in Figure 2. (2) The ring B of **1** was elucidated from an analysis of the long-range couplings in HMBC in the same manner as described above. Long-range couplings from the hydroxyl proton OH-8 (δ 9.41) to the sp^2 carbons C-7 (δ 116.3) and C-9 (δ 116.8) and the sp^2 oxygenated carbon C-8 (δ 148.7) and from the methyl proton CH_3-9 (δ 2.15) to C-8, C-9 and the sp^2 oxygenated carbon C-9a (δ 142.4) were observed. Further long-range couplings from the sp^2 methine proton H-2′′ (δ 5.38) to the methyl carbon C-4′′ (δ 17.3), from the methyl proton H_3-3′′ (δ 1.76) to the sp^2 carbon C-1′′ (δ 129.8) and from the methyl proton H_3-4′′ (δ 1.84) to C-1′′ and the sp^2 methine carbon C-2′′ (δ 127.0) suggested the presence of an isobutyl group containing the partial structure II. This isobutyl group was connected to the quaternary carbon C-6 (δ 134.0) by the observation of ^3J cross peaks from H-2′′ and H_3-4′′ to C-6 in HMBC experiments. A comparison of the NMR data between **1** and **2** revealed that the aromatic ^{13}C chemical sift values were very similar, whereas the sp^2 methine carbon at C-7 in **2** was replaced by a sp^2 fully substituted carbon in **1**, indicating that ring B in **1** had a hexasubstituted benzene ring and the aromatic proton at C-7 of **2** was substituted by a chlorine atom in **1** (Figure 2). (3) The molecular formula and degrees of unsaturation of **1** indicated that the aromatic rings A and B were connected via an ether linkage between C-4a and C-5a and an ester bridge between C-9a and C-11, suggesting a depsidone skeleton with a central seven-membered ring C. Taken together, the structure of **1** was elucidated as shown in Figure 1, that fulfilled the molecular formula and degrees of unsaturation.

Inhibition of SOAT isozymes using SOAT1- and SOAT2-CHO cells

Cell-based assay. The effects of **1** to **6** on cholesteryl ester (CE) synthesis were evaluated in a cell-based assay using SOAT1- and SOAT2-CHO cells. As shown in Table 3, **1** to **4** inhibited SOAT1 and SOAT2 isozymes with analogous IC_{50} values (2.0–16 μM), giving selectivity index (log (IC_{50} for SOAT1)/(IC_{50} for SOAT2)) values (−0.15 to 0.88) ranging between −1.00 and +1.00 (dual-type

Table 2 ^{1}H and ^{13}C NMR chemical shifts of 1 and 2 in DMSO-d_6

	1						*2*					
Position	δ_C	*mult*	δ_H		*mult*	*J (Hz)*	δ_C	*mult*	δ_H		*mult*	*J (Hz)*
1	148.4	s					147.6	s				
2	111.9	d	6.55	1H	s		111.9	d	6.53	1H	s	
3	159.9	s					159.4	s				
4	113.3	s					113.4	s				
4a	161.3	s					161.6	s				
5a	141.9	s					141.7	s				
6	134.0	s					135.7	s				
7	116.3	s					111.0	d	6.39	1H	s	
8	148.7	s					152.5	s				
9	116.8	s					114.8	s				
9a	142.4	s					143.2	s				
11	163.0	s					163.5	s				
11a	110.0	s					110.9	s				
1'	135.7	s					133.5	s				
2'	123.9	d	5.34	1H	dq	1.5, 7.0	123.8	d	5.32	1H	dq	1.5, 6.7
3'	14.1	q	1.64	3H	dd	1.5, 7.0	13.8	q	1.64	3H	dd	1.5, 6.7
4'	17.5	q	1.77	3H	d	1.5	17.8	q	1.77	3H	d	1.5
1''	129.8	s					135.6	s				
2''	127.0	d	5.38	1H	dq	1.5, 7.0	125.1	d	5.46	1H	dq	1.5, 6.7
3''	13.8	q	1.76	3H	dd	1.5, 7.0	14.1	q	1.72	3H	dd	1.5, 6.7
4''	17.3	q	1.84	3H	d	1.5	17.4	q	1.97	3H	d	1.5
4-Me	8.5	q	2.04	3H	s		8.3	q	2.02	3H	s	
9-Me	10.1	q	2.15	3H	s		9.0	q	2.03	3H	s	
3-OH			10.54	1H	s				10.42	1H	s	
8-OH			9.41	1H	s				9.65	1H	s	

The ^{13}C (100 MHz) and ^{1}H (400 MHz) spectra were taken on XL-400 NMR system (Agilent) in DMSO-d_6, and solvent peak was used as an internal standard at 2.49 ppm for ^{1}H-NMR and at 39.5 ppm for ^{13}C-NMR.

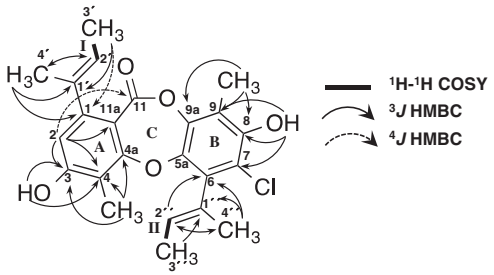


Figure 2 Key correlations in ^{1}H–^{1}H COSY and heteronuclear multiple bond correlation (HMBC) spectra of 7-chlorofolipastatin (**1**).

inhibition).[12] Both **5** and **6** also showed very weak inhibition (40–50% inhibition at 23–25 μM) in both SOAT1- and SOAT2-CHO cells.

Enzyme assay. The inhibition of SOAT by **1** to **6** was evaluated in the enzyme assay using microsomes prepared from SOAT1- and SOAT2-CHO cells. As shown in Table 3, the IC_{50} and selectivity index values of **1** to **6** in the enzyme assay were consistent with those in the cell-based assay. Depsidones with no or one chloride in their molecules (**1** to **4**) exhibited dual-type SOAT inhibition, whereas those with two chlorides (**5** and **6**) had decreased inhibitory activity.

Inhibition of CE synthesis in mouse peritoneal macrophages

The effects of **1** to **6** on CE synthesis (synthesized by SOAT1) were investigated in mouse peritoneal macrophages according to our established method.[19] CE accumulation in macrophages promotes atherosclerosis in arterial walls.[20] Compounds **1** to **4** inhibited CE synthesis with IC_{50} values of 6.8, 12 and 19 μM, respectively (Table 3), consistent with those in SOAT1-CHO cells.

Antimicrobial activity

The antimicrobial activities of **1** to **6** (10 μg per disk), as determined by the agar diffusion assay, are summarized in Table 4. All compounds exhibited weak antimicrobial activity against the Gram-positive bacteria, *Bacillus subtilis*, *Staphylococcus aureus* and *Micrococcus luteus*, but did not affect the growth of the Gram-negative bacteria, *Escherichia coli* and *Pseudomonas aeruginosa* or yeast *Candida albicans*.

Conclusion

In the present study, a new chlorinated depsidone, 7-chlorofolipastatin (**1**), was isolated together with five known depsidones, **2** to **6**, from the culture broth of a marine-derived *Aspergillus* strain. Compounds **1** to **4** moderately inhibited SOAT1 and SOAT2 isozymes (moderate dual-type SOAT inhibition).

METHODS

General

Various NMR spectra were obtained using 400 MHz spectrometer (Agilent Technologies, Santa Clara, CA, USA). FAB-MS spectra were recorded on a JMS-700 Mstation (JEOL, Tokyo, Japan). Optical rotations were measured with a digital polarimeter (DIP-1000; JASCO, Tokyo, Japan). UV spectra were recorded on an 8453 UV-visible spectrophotometer (Agilent Technologies). IR

Table 3 Effect of 1 to 6 on SOAT1 and SOAT2 activities in cell-based and enzyme-based assays

	IC_{50} (μM) for CE synthesis						
	Cell-based assay[b]				*Enzyme-based assay*[b]		
Compound	*SOAT1*	*SOAT2*	*SI*[a]	*Macrophage*	*SOAT1*	*SOAT2*	*SI*
1	3.2	4.5	−0.15	6.8	6.5	19	−0.47
2	9.6	2.0	0.68	12	10	6.6	0.18
3	16	2.1	0.88	19	27	10	0.43
4	>27	6.8	>0.60	>27	>69	28	>0.39
5	>25	>25	—	>25	>63	>63	—
6	>23	>23	—	>23	>58	>58	—

Abbreviations: CE, capillary electrophoresis; SI, selective index; SOAT, sterol *O*-acyltransferase.
[a]SI = log(IC_{50} for SOAT1)/(IC_{50} for SOAT2).
[b]*N* = >3.

Table 4 Antimicrobial activity of 1 to 6

	Inhibition zone (mm)					
Microorganism	***1***	***2***	***3***	***4***	***5***	***6***
Bacillus subtilis PCI219	8	9	—	—	10	8
Staphylococcus aureus FDA209P	—	8	8	9	16	9
Micrococcus luteus KB212	7	8	15	11	11	14
Escherichia coli JM109	—	—	—	—	—	—
Pseudomonas aeruginosa IFO12689	—	—	—	—	—	—
Candida albicans ATCC90029	—	—	—	—	—	—

Antimicrobial activity was measured by the agar diffusion method using paper disks (6 mm thin disk).

spectra were recorded on a Fourier transform an FT-710 spectrometer (Horiba, Kyoto, Japan).

Materials

The [1-^{14}C]Oleic acid (1.85 GBq mmol^{-1}) was purchased from PerkinElmer (Waltham, MA, USA). Fetal bovine serum was purchased from Biowest (Nuaille, France). Dulbecco's modified Eagle's medium and Hank's buffered salt solution were purchased from Nissui Pharmaceutical (Tokyo, Japan). GIT medium was from Nippon Seiyaku (Tokyo, Japan). Penicillin (10 000 units ml^{-1}), streptomycin (10 000 mg ml^{-1}) and glutamine (200 mm) solution were from Invitrogen (Carlsbad, CA, USA). Phosphatidylcholine, phosphatidylserine, dicetylphosphate, cholesterol, Ham's F-12 medium, malachite green oxalate and BFA_1 were purchased from Sigma-Aldrich (St Louis, MO, USA). Perchloric acid and Triton X-100 were purchased from Wako (Osaka, Japan).

Fungal strain and identification

The fungal strain NKH-007 was isolated from soil that was collected from the Suruga Bay, Japan (138°18.1207′E, 34°22.4813′N) at a depth of 331 m on 15 July 2013. In a BLAST search from the NCBI (National Center for Biotechnology Information), NKH-007 had 100% similarity with the rDNA sequence of the internal transcribed spacer region of *Aspergillus ungui* SRRC 344 (GenBank accession number AY373872).[21] Therefore, the strain NKH-007 was identified with *Aspergillus ungui*.

Fermentation of *Aspergillus ungui* NKH-007

A loopful of spores of strain NKH-007 was inoculated into 100 ml seed medium consisting of 2.4% potato dextrose broth (Becton Dickinson and Company, Franklin Lakes, NJ, USA) and 0.1% agar (adjusted to pH 6.0 before sterilization) in a 500-ml Erlenmeyer flask. The inoculated Erlenmeyer flask was incubated in a rotary shaker (200 r.p.m.) at 27 °C for 5 days to obtain the seed culture. In order to produce **1**, the culture was initiated by transferring 1 ml seed culture into each of six 500-ml culture bottles (AS ONE, Osaka, Japan) containing 100 ml production medium (2.4% potato dextrose broth, adjusted to pH 6.0 before sterilization). Fermentation was carried out under static conditions at 27 °C for 14 days.

Structural determination of 2 to 6

Using spectral data including ^{1}H NMR, ^{13}C NMR and MS, and the search results of SciFinder Scholar, **2** to **6** were identified as the known depsidones folipastatin (**2**), unguinol (**3**), 2-chlorounginol (**4**), 2,7-dichlorounginol (**5**) and nornidulin (**6**), respectively (Figure 1).

Cell culture

Two cell lines, CHO cells expressing SOAT1 and SOAT2 isozymes of African green monkey (SOAT1- and SOAT2-CHO cells, respectively),[13] were kind gifts from Dr LL Rudel (Wake Forest University, Winston-Salem, NC, USA). Cells were maintained as described previously.[12]

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

Assays for SOAT1 and SOAT2 activities using SOAT1- and SOAT2-CHO cells were carried out by our established method.[12] Briefly, SOAT1- or SOAT2-CHO cells (1.25×10^5 cells in 250 μl of medium) were cultured in a 48-well plastic microplate in the culture medium described above and allowed to recover overnight at 37 °C in 5% CO_2. The assays were conducted with cells that were at least 80% confluent. Following overnight recovery, the test sample (2.5 μl MeOH solution) and [1-^{14}C]oleic acid (5 μl 10% EtOH/phosphate-buffered saline solution, 1 nmol, 1.85 KBq) were added to each culture. The medium was removed after a 6-h incubation at 37 °C in 5% CO_2, and the cells in each well were washed twice with phosphate-buffered saline. The cells were lysed by adding 0.25 ml of 10 mM Tris-HCl (pH 7.5) containing 0.1% (w/v) sodium dodecyl sulfate, and [^{14}C]CE was analyzed with a BAS2000 analyzer (Fuji Film, Tokyo, Japan). In this cell-based assay, [^{14}C]CE was produced by the reaction of SOAT1 or SOAT2. SOAT inhibitory activity (%) was defined as ([1-^{14}C]CE-drug/[^{14}C]CE-control) × 100. The IC_{50} value was defined as the drug concentration that inhibited biological activity by 50%.

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

SOAT1 and SOAT2 activities were determined using microsomes prepared from SOAT1- and SOAT2-CHO cells as the enzyme source.[12] Briefly, an assay mixture containing 2.5 mg ml^{-1} bovine serum albumin in buffer A and [1-^{14}C]oleoyl-CoA (20 μM, 3.7 kBq) together with a test sample (added as a 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 for 5 min. The reaction was started by adding [1-^{14}C]oleoyl-CoA and stopped by adding 1.2 ml of $CHCl_3$/MeOH (2:1). The product [^{14}C]CE was extracted by the method of Bligh and Dyer.[22] 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.

Assay for CE synthesis in mouse peritoneal macrophages

The assay for CE synthesis from [^{14}C]oleic acid was carried out according to a previously described method.[19] Briefly, mouse peritoneal macrophages (5.0×10^5 cells per 0.25 ml of medium B (containing Dulbecco's modified Eagle's medium supplemented with 8.0% (v/v) lipoprotein-deficient serum, penicillin (100 units ml^{-1}) and streptomycin (100 mg ml^{-1})) were cultured in each well of a 48-well plastic microplate with a test compound (in 2.5 μl of CH_3CN) and liposomes (10.0 μl, 1.0 μmol phosphatidylcholine, 1.0 μmol phosphatidylserine, 0.20 μmol dicetylphosphate and 1.5 μmol cholesterol, suspended in 1.0 ml of 0.30 M glucose) together with [^{14}C]oleic acid (5.0 μl (1.85 kBq) in 10% ethanol/phosphate-buffered saline solution). Following a 14-h incubation, cellular lipids were extracted to measure the radioactivity of [^{14}C]CE according to the same method as described above.

Antimicrobial activity

The antimicrobial activity of a sample against six species of microorganism was measured by the agar diffusion method using paper disks. Media for microorganisms were as follows: nutrient agar (Sanko Junyaku, Tokyo, Japan) for *B. subtilis* PCI219, *S. aureus* FDA209P, *M. luteus* KB212, *E. coli* JM109 and *P. aeruginosa* IFO12689, and medium composed of 1.0% glucose, 0.50% yeast extract and 0.80% agar for *C. albicans* ATCC90029. A paper disk (i.d. 6 mm; Toyo Roshi Kaisha, Tokyo, Japan) containing a sample (10 μg) was placed on the agar plate. Bacteria were incubated at 37 °C for 24 h. *C. albicans* was incubated at 27 °C for 48 h. Antimicrobial activity was expressed as the diameter (mm) of the inhibitory zone.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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