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ORIGINAL ARTICLE

Clonoamide, a new inhibitor of sterol *O*-acyltransferase, produced by *Clonostachys* sp. BF-0131

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A new compound designated as clonoamide was isolated from a culture broth of the fungus *Clonostachys* sp. BF-0131 by solvent extraction, Diaion HP20 column chromatography, octadecylsilyl column chromatography and preparative HPLC as an inhibitor of sterol *O*-acyltransferase (SOAT). The structure of clonoamide was elucidated as 2-oxo-9*E*,11*E*-tridecandienyl acetamide by various spectral analyses including NMR. The compound inhibited SOAT1 and SOAT2 isozymes with IC_{50} values of 39 and 110 μM, respectively, in a cell-based assay using SOAT1- and SOAT2-expressing Chinese hamster ovary cells.
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INTRODUCTION

Sterol *O*-acyltransferase (SOAT, also known as acyl-CoA:cholesterol acyltransferase, EC 2.4.1.26), an endoplasmic reticulum membrane protein, catalyzes the synthesis of cholesteryl ester (CE) from free cholesterol and long-chain fatty acyl-CoA. SOAT has been considered as a potential target for post-statin drug development. Although statins, inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase, have been clinically used as powerful cholesterol-lowering agents, the risk of complications and death from cardiovascular events by statin treatment is reduced by only 30%.[1] Recent molecular biological studies revealed the existence of two distinct SOAT isozymes in mammals, SOAT1 and SOAT2.[2–5] SOAT1 is ubiquitously expressed in almost all tissues and cells, such as sebaceous glands, steroidogenic tissues and macrophages, whereas SOAT2 is expressed predominantly in the liver (hepatocytes) and intestine.[4] In the 2000s, the development of three SOAT inhibitors (avasimibe,[6] pactimibe[7] and K-604[8]) failed in clinical trials. The first two inhibitors inhibited both SOAT1 and SOAT2 (dual-type inhibitors), while the last one selectively inhibited SOAT1 (SOAT1-selective-type inhibitor). After that, several lines of evidence[9–11] indicated that SOAT2 will be a promising target for post-statin drug development.[12] Accordingly, we have focused on the discovery of SOAT2-selective inhibitors.[13]

During our screening for SOAT2 inhibitors from microorganisms,[14] a new compound named clonoamide (Figure 1) was isolated from the culture broth of *Clonostachys* sp. BF-0131 in a cell-based assay using SOAT2-expressing Chinese hamster ovary (SOAT2-CHO) cells. Unfortunately, the compound was found to inhibit both SOAT1 and SOAT2 moderately. In this study, the fermentation, isolation, structural elucidation and biological properties of clonoamide are described.

RESULTS

Fermentation

Fungal strain BF-0131 was isolated from a soil sample collected at Arisugawanomiya Memorial Park in Tokyo, Japan. In a BLAST search, the rDNA sequence of the internal transcribed spacer region showed high levels of similarity to the genus *Clonostachys*. Therefore, the producing strain was named *Clonostachys* sp. BF-0131. The slant culture of the strain grown on 2.4% potato dextrose agar (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of seed medium (2.4% potato dextrose broth (Becton, Dickinson and Company) and 0.10% agar, pH 6.0). The flask was shaken on a rotary shaker at 27 °C for 3 days. The seed culture (3.0 ml) was inoculated into a culture bottle containing the production medium (50 g of Italian rice (Japan Europe Trading Co. Ltd., Tokyo, Japan), 1.2% potato dextrose broth, 1.0% malt extract, 1.0% glucose and 0.050% peptone, pH 6.0). The fermentation was carried out under static conditions at 27 °C for 14 days.

Isolation

The 14-day-old culture broth (1.5 kg) of the fungus was treated with 70% EtOH (5.0 l). After the mixture had been filtered and concentrated to remove EtOH, the residual aqueous solution (900 ml) was applied to a Diaion HP20 column (150 ml; Mitsubishi Chemical, Tokyo, Japan), and materials were eluted stepwise with H_2O and MeOH (300 ml each). The MeOH fraction including the active principle was concentrated under reduced pressure to give a brown material (1.3 g). This material was dissolved in a small amount of MeOH and applied to an octadecylsilyl column (39 g; Fuji Silysia Chemical, Aichi, Japan). Materials were eluted stepwise with 0, 20, 40, 60, 80 and 100% CH_3CN (100 ml each). The 80% CH_3CN fraction

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including the active principle was concentrated under reduced pressure to give a brown material (46 mg). Clonoamide was finally purified by preparative HPLC under the following conditions: column, PEGASIL ODS SP100, 20×250 mm (Senshu Scientific Co., Tokyo, Japan); mobile phase, 35-min gradient from 70% CH_3CN to 80% CH_3CN; detection, UV at 210 nm; flow rate, 8.0 ml min^{-1}. Under these conditions, clonoamide was eluted as a peak with a retention time of 28 min. The fraction was concentrated under reduced pressure to give pure clonoamide (4.6 mg) as a white powder.

Structural elucidation of clonoamide

The physico-chemical properties of clonoamide are summarized in Table 1. The molecular formula was determined to be $C_{15}H_{25}NO_2$ on the basis of HR-FAB-MS measurement. The UV spectrum of clonoamide had an absorption maximum at 225 nm. The IR spectrum showed the absorption maxima at 3311, 1711 and 1654 cm^{-1}, suggesting the presence of amino, ketone and amide moieties, respectively. The ^{13}C NMR spectrum (in $CDCl_3$) showed 15 resolved signals, which were classified by the DEPT spectrum into two methyl carbons, 7 sp^3 methylene carbons including one methylene carbon connected to a hetero atom, 4 sp^2 methine carbons and two quaternary carbons including carbonyl and ketone carbons. The connectivity of proton and carbon atoms was established by the HMQC spectrum, as shown in Table 2. Analysis of the ^1H–^1H COSY spectrum gave the two partial structures **I** (C-1 to NH) and **II** (C-3 to C-13) drawn as the bold lines in Figure 2. Regarding the stereochemistry of the conjugated double bonds in **II**, the coupling constants of 9-H (14.0 Hz), 10-H (14.0 Hz), 11-H (14.0 Hz) and 12-H (14.0 Hz) indicated that they are assigned as 9*E* and 11*E*. Analysis of the ^1H–^{13}C long-range couplings of ^2J and ^3J observed in the HMBC experiments gave the following information. The cross peaks from 1-H_2 (δ 4.42), 3-H_2 (δ 2.43) and 4-H_2 (δ 1.61) to C-2 (δ 205.5) and from 3-H_2 to C-1 (δ 49.2) indicated the presence of a 1-amino-2-oxo-9*E*, 11*E*-tridecadiene moiety containing the partial structures **I** and **II**. Furthermore, the cross peaks from 1-H_2 and 2′-H_3 (δ 2.04) to C-1′ (δ 170.0) supported that the amino residue was acetylated. Taking these findings together, the structure of clonoamide was elucidated as 2-oxo-9*E*,11*E*-tridecandienyl acetamide, which fulfilled the molecular formula and the degrees of unsaturation.

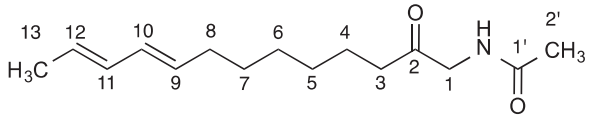

Figure 1 Structure of clonoamide.

Table 1 Physico-chemical properties of clonoamide

Appearance	White powder
Molecular weight	251
Melecular formula	$C_{15}H_{25}NO_2$
HR-FAB-MS (*m/z*)	
Calcd	274.1765 $(M+Na)^+$
Found	274.1783 $(M+Na)^+$
UV λ_{max}^{MeOH} (logε)	225 (4.0)
IR ν_{max}^{KBr} cm^{-1}	3311, 2921, 2849, 1711, 1654, 1561, 1432

Biological properties

Inhibition of CE synthesis in SOAT1 and SOAT2-CHO cells. The effect of clonoamide on SOAT1 and SOAT2 isozymes was evaluated in a cell-based assay by using SOAT1- and SOAT2-CHO cells. As shown in Figure 3a, clonoamide dose dependently inhibited CE synthesis in both SOAT1- and SOAT2-CHO cells with IC_{50} values of 39 and 110 μM, respectively. It showed no cytotoxic effect at 120 μM by the MTT assay.

To confirm the inhibitory activity against SOAT isozymes, the effect of clonoamide on SOAT1 and SOAT2 was investigated in an enzyme assay by using microsomal fractions prepared from the respective cells. As shown in Figure 3b, clonoamide inhibited SOAT1 and SOAT2 activities with IC_{50} values of 37 and 130 μM, respectively. These results are consistent with those in a cell-based assay.

Inhibition of CE synthesis in mouse peritoneal macrophages. Inhibition of SOAT1 activity by clonoamide was confirmed in our established assay by using mouse peritoneal macrophages, which exclusively express SOAT1. As shown in Figure 3c, clonoamide inhibited CE synthesis with IC_{50} value of 16 μM and showed no cytotoxic effect at 120 μM by the alamar blue assay.

Antimicrobial activity. No antimicrobial activity of clonoamide (10 μg per paper disk) against *Bacillus subtilis*, *Staphylococcus aureus*, *Micrococcus luteus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans* was observed by the agar diffusion assay using paper disks.

Table 2 ^1H and ^{13}C NMR chemical shifts of clonoamide

Position	*δ_C (p.p.m.)* [a], *multi*	*δ_H (p.p.m.)* [b], *multi, J in Hz*
1	49.2, t	4.42, d, 4.5
2	205.5, s	—
3	40.3, t	2.43, t, 8.0
4	23.6, t	1.61, m
5	29.1, t	1.28, m
6	28.7, t	1.28, m
7	28.9, t	1.36, m
8	32.4, t	2.03, br. dd, 6.5, 14.0
9	131.8, d	5.52, ddd, 6.5, 6.5, 14.0
10	131.6, d	6.01, dd, 12.0, 14.0
11	130.4, d	5.98, dd, 12.0, 14.0
12	126.8, d	5.57, dq, 7.3, 14.0
13	17.9, q	1.72, d, 7.3
1'	170.0, s	—
2'	22.9, q	2.04, s
NH	—	6.23, br. s

^1H and ^{13}C NMR spectra were obtained at 600 and 150 MHz, respectively and recorded in $CDCl_3$.
[a]Chemical shifts are shown with reference to $CDCl_3$ as δ 77.0.
[b]Chemical shifts are shown with reference to $CDCl_3$ as δ 7.26.

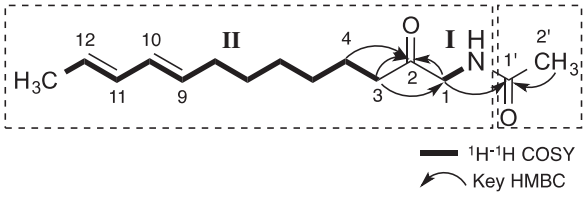

Figure 2 Key cross peaks observed in ^1H–^1H COSY and ^1H–^{13}C HMBC experiments of clonoamide.

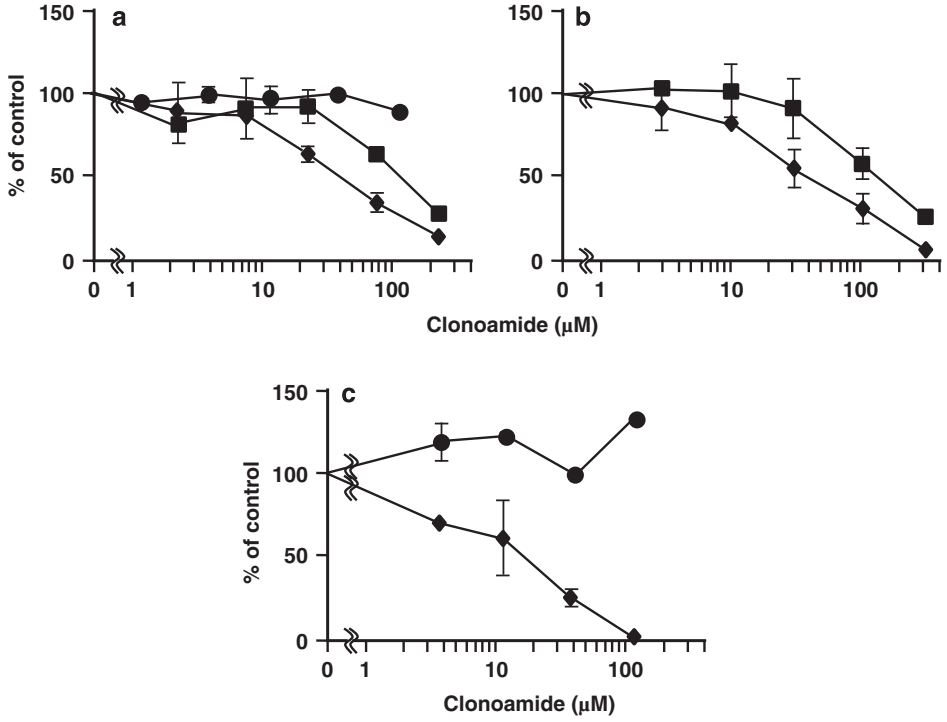

Figure 3 Effects of clonoamide on cholesteryl ester synthesis in (**a**) SOAT1- and SOAT2-CHO cells, (**b**) microsomes prepared from SOAT1- and SOAT2-CHO cells and (**c**) mouse peritoneal macrophages. SOAT1 (**a** and **b**) and CE (**c**) (♦), SOAT2 (■) and cell viability (●).

DISCUSSION

Clonoamide, produced by the fungus *Clonostachys* sp. BF-0131, was first discovered as a moderate inhibitor of CE synthesis in SOAT2-CHO cells. From its selectivity test in inhibition toward the two isozymes, the compound inhibited CE synthesis in both SOAT1-(IC_{50}, 39 μM) and SOAT2-CHO cells (IC_{50}, 110 μM), as shown in Figure 3a. Furthermore, good consistent inhibition by the compound was observed in SOAT1 (IC_{50}, 37 μM) and SOAT2 (IC_{50}, 130 μM) enzyme assays (Figure 3b). These data indicated that the target molecules of clonoamide are SOAT isozymes, although it appears to prefer SOAT1 to SOAT2. Inhibition of SOAT1 by the compound (IC_{50}, 16 μM) was confirmed in the assay using intact mouse peritoneal macrophages (Figure 3c).

The structure of clonoamide was completely elucidated. Chemically synthetic 1-acetamide-2-tridecanone[15] and 1-acetamidetridecane,[16] structurally related to clonoamide, are already known, but the biological activities have not been reported. In this study, clonoamide was found to inhibit SOAT1 and SOAT2 isozymes. A number of synthetic SOAT inhibitors were reported. One of the important groups is an amide type, such as CI-976,[17] HL-004[18] and F-12511.[19] They consist of an amide core with a long acyl chain. Therefore, clonoamide can be classified into this type. Our group re-evaluated the selectivity of known SOAT inhibitors toward the two isozymes in cell-based assay or enzyme assay by using SOAT1- and SOAT2-CHO cells.[14,20] Through the experiments, we classified SOAT inhibitors into three types from the selective index (SI) values, which are defined as log (IC_{50} for SOAT1/IC_{50} for SOAT2);[13] inhibitors with $SI > +1$, $+1 \geqq SI \geqq -1$ and $-1 > SI$ are SOAT2-selective type, dual type and SOAT1-selective type, respectively. Most inhibitors including avasimibe and pactimibe belong to the dual type. Synthetic K-604 and Wu-V-23 and fungal beauveriolides I and III[21] belong to the SOAT1-selective type. Pyripyropenes are a rare example belonging to the SOAT2-selective type. According to the definition, clonoamide with SI of -0.54 is classified as a dual-type inhibitor. Our group will continue to search for SOAT2-selective inhibitors of microbial origin, which are expected to have potential as post-statin drugs.[12,13]

MATERIALS AND METHODS

General

Various NMR spectra were obtained using an INOVA 600 MHz spectrometer (Agilent Technologies, Santa Clara, CA, USA). FAB-MS spectra were recorded on a mass spectrometer (JMS-700 Mstation; JEOL, Tokyo, Japan). UV spectra were recorded on a spectrophotometer (Beckman DU640 spectrophotometer; Beckman Coulter, Inc., Fullerton, CA, USA). IR spectra were recorded on a Fourier transform infrared spectrometer (FT-710; Horiba Ltd, Kyoto, Japan).

Material

[1-^{14}C]Oleic acid (1.85 GBq mmol^{-1}) and [1-^{14}C]oleoyl-coenzyme A (1.85 GBq mmol^{-1}) were 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 Co. (Tokyo, Japan). GIT medium was from Nippon Seiyaku Co. (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, fatty acid-free bovine serum albumin and MEM vitamin solution 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 SOAT1 and SOAT2 isozymes of African green monkey (SOAT1- and SOAT2-CHO cells, respectively),[22] were kind gifts from Dr Lawrence L. Rudel (Wake Forest University, Winston Salem, NC, USA) and were cultured by a method described previously.[20]

Assays for SOAT1 and SOAT2 activities using SOAT1- and SOAT2-CHO cells

Assays for SOAT1 and SOAT2 activities using SOAT1- and SOAT2-CHO cells were carried out by our established method.[21] Briefly, SOAT1- 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 medium A containing Ham's F-12 medium supplemented with 10% heat-inactivated fetal bovine serum, MEM vitamins, penicillin (100 units ml^{-1}), streptomycin (100 units ml^{-1}) and geneticin (300 μg ml^{-1}) and allowed to recover overnight at 37 °C in 5.0% CO_2. The assays were carried out under at least 80% confluent cell conditions. Following overnight recovery, a test sample (in 2.5 μl of MeOH) and [^{14}C]oleic acid (1 nmol, 1.85 kBq in 5.0 μl of 10% EtOH/phosphate-buffered saline) were added to each well of the culture. After 6 h of incubation at 37 °C in 5.0% CO_2, the cells in each well were washed twice with phosphate-buffered saline and lysed by adding 0.25 ml of 10 mM Tris-HCl (pH 7.5) containing 0.10% (w/v) SDS, and the cellular lipids were extracted by the method of Bligh and Dyer.[23] After concentrating the organic solvent, the total lipids were separated on a TLC plate (silica gel F254, 0.5 mm thick, Merck, Kenilworth, NJ, USA), which was analyzed with a bioimaging analyzer (FLA-7000; Fujifilm, Tokyo, Japan) to measure the amount of [^{14}C]CE. The IC_{50} value was defined as a drug concentration causing 50% inhibition of biological activity.

Preparation of microsomes from SOAT1- or SOAT2-CHO cells

SOAT1- 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 (5.0 mg ml^{-1} aprotinin, 5.0 mg ml^{-1} leupeptin, 5.0 mg ml^{-1} pepstatin and 0.50 mM phenylmethanesulfonyl fluoride) in a teflon homogenizer. The microsomal fraction was pelleted by centrifugation at 100 000 *g* for 1 h at 4 °C, resuspended in the same buffer at a concentration of 5.0 mg protein ml^{-1} and stored at − 80 °C until use.

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

SOAT1 and SOAT2 activities were determined by using microsomes prepared as described above as the enzyme source.[24] Briefly, an assay mixture containing 2.5 mg ml^{-1} fatty acid-free bovine serum albumin in buffer A and [^{14}C]oleoyl-CoA (3.7 kBq) together with a sample (added as a 5.0 μl MeOH 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 [^{14}C]oleoyl-CoA, and stopped by adding 1.2 ml of $CHCl_3$:MeOH (2:1). The total lipids were extracted to measure the radioactivity of [^{14}C]CE according to the same method as described above.

MTT assay

Assay for cytotoxic activity using CHO cells was carried out by our established method.[25] Briefly, cytotoxicity of a sample to CHO cells was measured by the colorimetric assay on MTT. CHO cells (5.0×10^4 cells in 100 μl) were added to each well of a 96-well microplate. A sample (1.0 μl in MeOH) was added to each well, and the cells were incubated for 12 h at 37 °C. MTT (10 μl of 5.5 mg ml^{-1} stock solution) and a cell lysate solution (90 μl, 40% *N*, *N*-dimethylformamide, 20% SDS, 2.0% CH_3COOH and 0.030% HCl) were added to each well, and the microplate was shaken for 2 h. The OD of each well was measured at 540 nm by using a microtiter-plate reader (Elx 808; BioTek Instruments, Winooski, VT, USA).

Assay for CE synthesis in mouse peritoneal macrophages

The assay for the synthesis of neutral lipid from [^{14}C]oleic acid was carried out according to a previously described method.[26] Briefly, mouse peritoneal macrophages (5.0×10^5 cells in 250 μl 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 MeOH) 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 14 h of incubation, cellular lipids were extracted to measure the radioactivity of [^{14}C]CE according to the same method as described above.

Alamar blue assay

The cytotoxicity of clonoamide to mouse macrophages after 14 h of incubation was measured by using alamar blue reagent according to the established method.[23]

Antimicrobial activity

Antimicrobial activity of a sample against six species of microorganisms was measured by the agar diffusion method by using paper disks. Media for microorganisms were as follows: nutrient agar (Sanko Junyaku Co., Ltd, Tokyo, Japan) for *B. subtilis* PCI219, *S. aureus* FDA209P, *M. luteus* KB212, *E. coli* JM109 and *P. aeruginosa* IFO12689, and a 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.

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