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ORIGINAL ARTICLE

Ceramidastin, a novel bacterial ceramidase inhibitor, produced by *Penicillium* sp. Mer-f17067

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Decrease of ceramide in the skin is one of the aggravating factors of atopic dermatitis. The skin is often infected by ceramidase-producing bacteria, such as *Pseudomonas aeruginosa*. The bacterial ceramidase then degrades ceramide in the skin. To develop anti-atopic dermatitis drugs, we searched for ceramidase inhibitors, which led to the discovery of ceramidastin, a novel inhibitor of bacterial ceramidase, from the culture broth of *Penicillium* sp. Mer-f17067. Ceramidastin inhibited the bacterial ceramidase with an IC_{50} value of $6.25 \, \mu g \, ml^{-1}$. Here we describe the isolation, physicochemical properties, structure determination and biological activity of ceramidastin.

INTRODUCTION

In the skin, ceramide forms a multilamelle structure in the stratum corneum and functions as a permeability barrier as well as a water retainer.[1] However, the content of ceramide is found to be decreased in lesions of atopic skin.[2] The decrease of ceramide induces dryness in the skin of patients with atopic dermatitis and compromises the permeability barrier, permitting the invasion of allergens or irritants.[3] Interestingly, such atopic skin is frequently colonized by ceramidase-producing bacteria.[4] Ceramidase is an enzyme that catalyzes the hydrolysis of the *N*-acyl linkage of ceramide to produce a free fatty acid and a sphingosine base. Ito and colleagues[5] have isolated a ceramidase-producing bacterium, *Pseudomonas aeruginosa* AN17, from the skin of patients with atopic dermatitis and cloned the gene encoding its ceramidase.[6] The hypothesis driving this study is that the ceramidase from colonizing bacteria contribute to the decrease of ceramide in atopic skin lesions, and that inhibiting bacterial ceramidase will prevent the decrease of ceramide in atopic skin and improve the disease. In the course of our screening for ceramidase inhibitors, we discovered ceramidastin, a novel inhibitor of bacterial ceramidase, from the culture broth of *Penicillium* sp. Mer-f17067. Here we describe the isolation, physicochemical properties, structure determination and biological activity of ceramidastin.

MATERIALS AND METHODS

Microorganism

Fungal strain *Penicillium* sp. Mer-f17067 was isolated from a soil sample collected from Iriomote Island, Okinawa prefecture, Japan. The fungal strain was cultured and kept on potato dextrose agar (Difco, Detroit, MI, USA). This strain has been deposited as NITE P-580 at the NITE Patent Microorganisms Depositary, Japan.

Taxonomic studies

The following media were used to identify the producing fungus: potato dextrose agar, 2% malt agar, oatmeal agar and Miura's medium (LcA). The formed colonies were observed after 1-week incubation at 25 °C. The colors used in this study were taken from Kornerup and Wanscher.[7]

Fermentation

Fungal strain *Penicillium* sp. Mer-f17067 grown on an agar slant was inoculated into 100 ml of a seed medium containing 2% potato starch, 1% glucose, 2% Soypro (J-Oil Mills Inc., Tokyo, Japan), 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$ and three glass beads, and cultured at 25 °C for 3 days on a rotary shaker at 220 r.p.m. In all, 1 ml of the seed culture was inoculated into a 500-ml flask containing 100 ml of a culture medium consisting of 5% maltose hydrate, 1.5% Pharmamedia (Traders Protein, Memphis, TN, USA), 1% malt extract, 0.5% ammonium sulfate, 1% mineral solution (solution of 2% $CoCl_2 \cdot 6H_2O$, 2% $CaCl_2$ and 2% $MgCl_2$; adjusted at pH 7 before sterilization) and cultured at 25 °C for 4 days on a rotary shaker at 220 r.p.m.

Analytical measurement

Melting points were obtained on a Yanagimoto micro melting point apparatus (Yanagimoto, Kyoto, Japan). Optical rotations were measured on a JASCO P-1030 polarimeter (JASCO, Tokyo, Japan). UV spectra were recorded on a Hitachi 228 A spectrometer (Hitachi, Tokyo, Japan). ^1H and ^{13}C NMR spectra were measured on a JEOL JNM A400 spectrometer (JEOL, Tokyo, Japan) using tetramethylsilane (TMS) as an internal standard. HRESI-MS spectra were measured with a JEOL JMS-T100LC spectrometer (JEOL).

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Ceramidase activity

Ceramidase activity was assessed according to the method used by Okino *et al.*,[5] with minor modifications. *P. aeruginosa* no. 12 was cultured in 3% Trypto-Soya Broth (Nissui Seiyaku, Tokyo, Japan) at 37 °C for 3 h on a rotary shaker at 100 r.p.m. In all, 15 μl of the seed culture was inoculated into 3 ml of a synthetic medium consisting of 0.05% NH_4Cl, 0.05% K_2HPO_4, 0.5% NaCl (adjusted at pH 7.2 before sterilization) with 0.05% taurodeoxycholate and 0.05% sphingo-myelin, and cultured at 30 °C for 24 h on a rotary shaker at 100 r.p.m. After filtration, the cultured supernatant was used as a source for bacterial ceramidase.

The activity of ceramidase was measured using 7-nitrobenz-2-oxa-1, 3-diazole-labeled *N*-dodecanoylsphingosine (C12-NBD-ceramide; Avanti Polar Lipids, Alabaster, AL, USA) as a substrate. Here, 10 μl of 10 μM C12-NBD-ceramide in 50 mM Tris-HCl (pH 8.5) was mixed with 10 μl of the bacterial ceramidase solution and incubated with ceramidastin at 37 °C for 2.5 h. The reaction was terminated by the addition of 100 μl of chloroform/methanol (2/1, v/v). The resulting solution was evaporated and resolved in 100 μl of chloroform/methanol (2/1, v/v). A sample of 20 μl was applied on a thin layer chromatography (TLC) plate, which was developed with chloroform:methanol:25% ammonia (90:20:0.5). The spot of C12-NBD-fatty acid released by the reaction of the enzyme on the TLC plate was quantified by an FLA5000 Image Reader (Fujifilm, Tokyo, Japan).

Atopic dermatitis model

We used NC/Nga female mice (6-week old; Charles River, Yokohama, Japan) as an atopic dermatitis model.[8,9] Hair was removed from the backside of the mice anesthetized with Nembutal. The hairless area was defatted by acetone:ether (1:1) and washed with distilled water. Then, 200 μl of 1/4 diluted ceramidase solution was applied on the defatted back. After 30 min, 200 μl of 1 mg ml^{-1} ceramidastin dissolved in distilled water was applied on the defatted back. The procedure of defatting and applications of ceramidase and ceramidastin were repeated twice a day for 5 days a week and continued until the end of the experiment. Forty days after the first treatment, the mouse was killed and the skin on the backside was excised. The skin was fixed in formalin solution and embedded in paraffin, cut into 4-μm sections and stained with toluidine blue.

Cytotoxicity

Cells were inoculated in 96-well plates at 5000 cells per well with ceramidastin and cultured for 2 days. The growth was determined using 3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St Louis, MO, USA).

RESULTS

Taxonomy of the producing strain Mer-f17607

Colonies on potato dextrose agar plates were 32–34 mm in diameter, floccose to velvet and grayish blue (23C-4) to orange (5A6-7). Colonies on MA were 30–32 mm in diameter, velvet, and olive (1F-8) to pale orange (5A-3). Colonies on oatmeal agar plates were 35–37 mm in diameter, floccose to velvet, and dark green (25F-7) to light orange (5A4-5). Colonies on LcA plates were 25–28 mm in diameter, velvet, and olive (1F-8) to pale orange (5A-3). Soluble pigment was not found in the culture on any media. Vegetative hyphae were formed in or on agar plates, colorless and smooth in surface with septate hyphae. Conidiophores were orthotropous in vegetative hyphae, colorless and smooth in surface with metulae at the apex. Metulae were cylindrical and phialides were needle-like. Conidia were phialoconidia, one-celled, subrounded to rounded, smooth in surface and connected to each other like a chain from a phialide. Sexual reproductive organs were not found when the culture was observed for 2 weeks. These cultural and morphological characteristics suggest that the strain belonged to the genus *Penicillium*. On the basis of 28S rDNA-D1/D2 gene sequence data, the strain formed a distinct phyletic line from any known species in the genus *Penicillium*. Therefore, we classified it as a strain of *Penicillium* called *Penicillium* sp.

Isolation procedure for ceramidastin

The culture broth (5 l) was filtered and the filtrate was passed through a DIAION HP20 column (500 ml) equilibrated with H_2O. After washing with H_2O (2 l) and 20% acetone (2 l), the active material was eluted with 75% acetone (2 l). The eluate was concentrated *in vacuo* to remove acetone and diluted with H_2O up to 2.2 l. After adjusting the pH to 8, the aqueous solution was extracted with BuOH. The aqueous layer was concentrated *in vacuo* to remove the residual BuOH and diluted with H_2O up to 2.6 l. After adjusting the pH to 3, the aqueous solution was extracted with EtOAc (1.3 l×2). The organic layer was concentrated *in vacuo* to give a brown material (1.5 g). A portion of the material was dissolved in a small volume of 20% acetonitrile containing 0.1% trifluoro acetic acid (TFA) and passed through an Inertsil ODS-3 column (20×250 mm) packed with the same solution. Active ingredients containing ceramidastin were eluted from the column using a linear gradient system (20–60% acetonitrile containing 0.1% TFA). The active ingredients containing ceramidastin were diluted with 10 volumes of H_2O and applied on an HP20 column. After washing the column with H_2O, elution was carried out using acetone to afford 245 mg of ceramidastin.

Physicochemical properties

The physicochemical properties of ceramidastin are summarized in Table 1. Ceramidastin was isolated as a white powder, which was soluble in water, MeOH, acetone and DMSO.

Structure determination

The molecular formula of ceramidastin was determined to be $C_{26}H_{34}O_{11}$ on the basis of HRESI-MS (Table 1) and ^{13}C NMR information. The ^{13}C NMR spectrum (DMSO-d_6) showed 26 discrete carbon signals, which were classified into one methyl, nine methylenes, eight methines, including two sp^2 and six sp^3 methines, four sp^2 quaternary carbons and four carbonyl carbons by analysis of DEPT spectra. The connectivity of proton and carbon atoms was established by the ^{13}C–^1H heteronuclear multiple quantum coherence (HMQC) spectrum. Four of five hydroxyl signals were detected in the ^1H NMR spectrum (Table 2). The connectivity of these hydroxyl moieties and relevant carbon atoms was established by the ^1H–^1H COSY and heteronuclear multiple bond connectivity (HMBC) experiments (Table 2 and Figure 2). The general features of ^1H and ^{13}C NMR spectra resembled those of rubratoxin A and B.[10,11] As shown by the bold lines in Figure 2, five partial structures, composed of I (-CH_2-CH=CH-), II (-CH_2-CH(CH_2-)-CH(OH)-), III (-CH-CH(OH)-), IV (-CH-OH) and V (-CH_2-CH_3), were deduced from the ^1H–^1H COSY spectra. The arrow lines show that the partial structure from C-6 to

Table 1 Physicochemical properties of ceramidastin

Appearance	White powder
Molecular formula	$C_{26}H_{34}O_{11}$
HRESI-MS (m/z)	
(Positive mode)	
Found	545.2047 (M+Na)$^+$
Calculated	545.1999 for $C_{26}H_{34}O_{11}Na$
(Negative mode)	
Found	521.2011 (M-H)$^-$
Calculated	521.2023 for $C_{26}H_{33}O_{11}$
Melting point	121–124 °C
UVλ$_{max}$ nm (H_2O)	End
UVλ$_{max}$ nm (0.01 N HCl)	275 (sh)
[α]$_D^{24}$ (ca. 0.5, H_2O)	+29°

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Table 2 The ^{13}C and ^{1}H NMR assignments of ceramidastin in DMSO-d_6

Position	^{13}C (mult.)	p.p.m.	^{1}H p.p.m. (mult., J(Hz))
1	61.7	(t)	3.90 (d, 5.0)
2	132.5	(d)	5.55 (dt, 15.3, 5.0)
3	126.5	(d)	5.65 (dt, 15.3, 7.0)
4	35.8	(t)	2.05 (m), 2.25 (m)
5	71.5	(d)	3.50 (ddd, 10.4, 7.2, 4.0)
5-OH			3.90 (br s)
6	78.0	(d)	3.20 (m)
6-OH			4.90 (br s)
7	36.0	(d)	1.85 (m)
8	24.1	(t)	2.35 (m), 2.62 (m)
9	143.8	(s)	
10	142.5	(s)	
11	63.0	(d)	5.28 (d, 10.5)
11-OH			5.95 (br s)
12	47.0	(d)	3.10 (m)
13	138.4	(s)	
14	148.0	(s)	
15	31.8	(t)	2.45 (m), 2.80 (m)
16	68.8	(d)	4.12 (m)
16-OH			4.84 (br s)
17	35.5	(t)	1.20 (m)
18	25.7	(t)	1.35 (m)
19	28.0	(t)	1.20 (m)
20	31.0	(t)	1.20 (m)
21	22.0	(t)	1.23 (m)
22	13.5	(q)	0.85 (t, 7.0)
23	165.8	(s)	
24	163.0	(s)	
25	167.0	(s)	
26	166.4	(s)	

Chemical shifts in p.p.m. from trimethylsilane (TMS) as an internal standard.
The ^{13}C and ^{1}H NMR were measured at 100 and 400 MHz, respectively.

Figure 2 1H-1H COSY and HMBC correlations in ceramidastin.
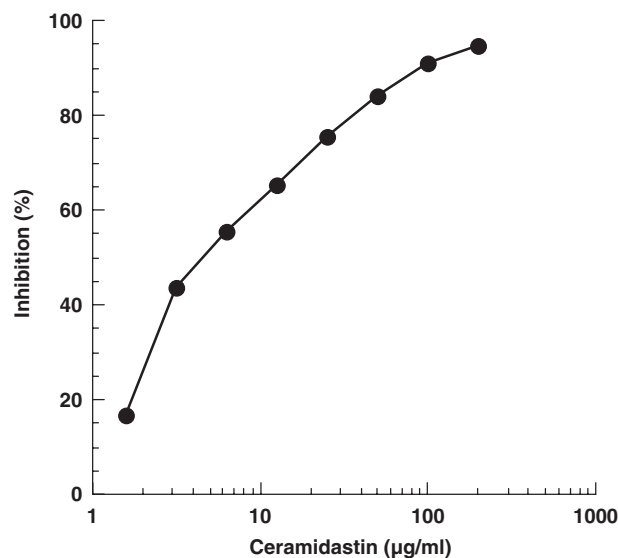
Figure 3 Effect of ceramidastin on *Pseudomonas* ceramidase. Values are means of duplicate determinations. Each SE is less than 10%.

those of reported rubratoxin B, suggesting the same stereochemistry between the two compounds. The remaining part (HO-CH$_2$-CH=CH-CH$_2$-CH(OH)-), including the partial structure of ceramidastin, was elucidated to connect C-6 by the HMBC correlation from H-4 as shown in Figure 2. The coupling constant between 2-H and 3-H (J=15.3 Hz) indicated that the configuration at C-2/C-3 is *E*. Consequently, the total structure of ceramidastin was elucidated as shown in Figures 1 and 2.

Inhibition of *Pseudomonas* ceramidase

The effect of ceramidastin on bacterial ceramidase was assessed according to the method used by Okino *et al.*,[5] with minor modifica-
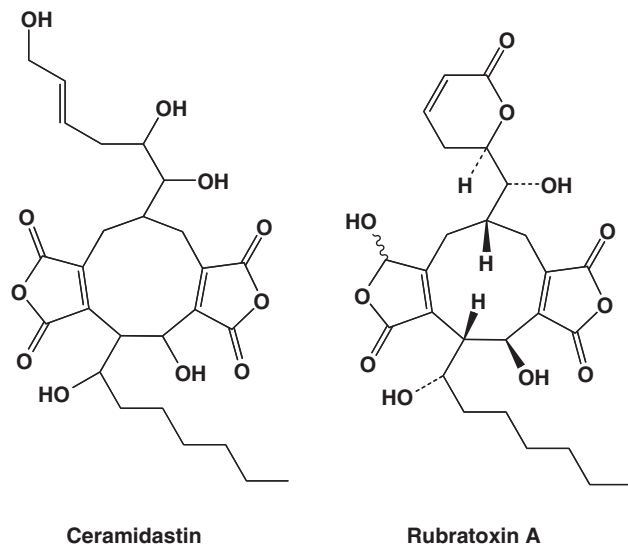

Figure 1 Structures of ceramidastin and rubratoxin A.

C-26 in ceramidastin, which is completely preserved in rubratoxin B, was deduced from the HMBC spectra. Furthermore, ^{1}H and ^{13}C chemical shifts and coupling constants of ceramidastin were very similar to
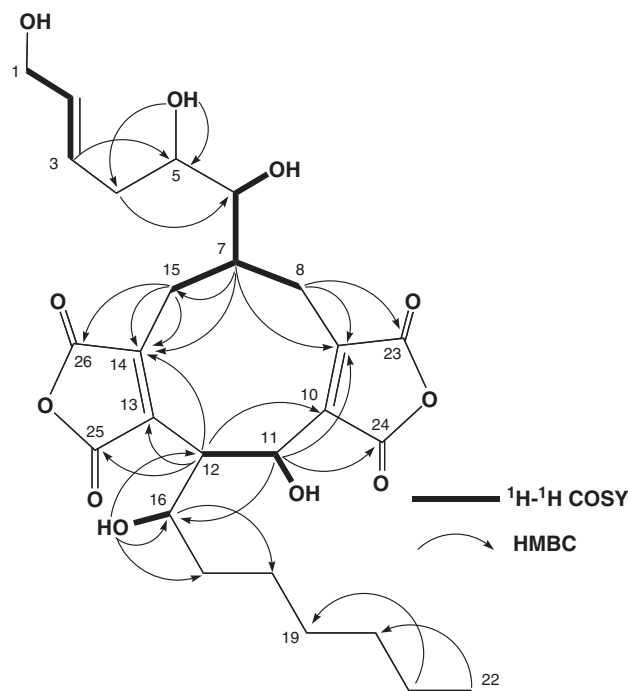
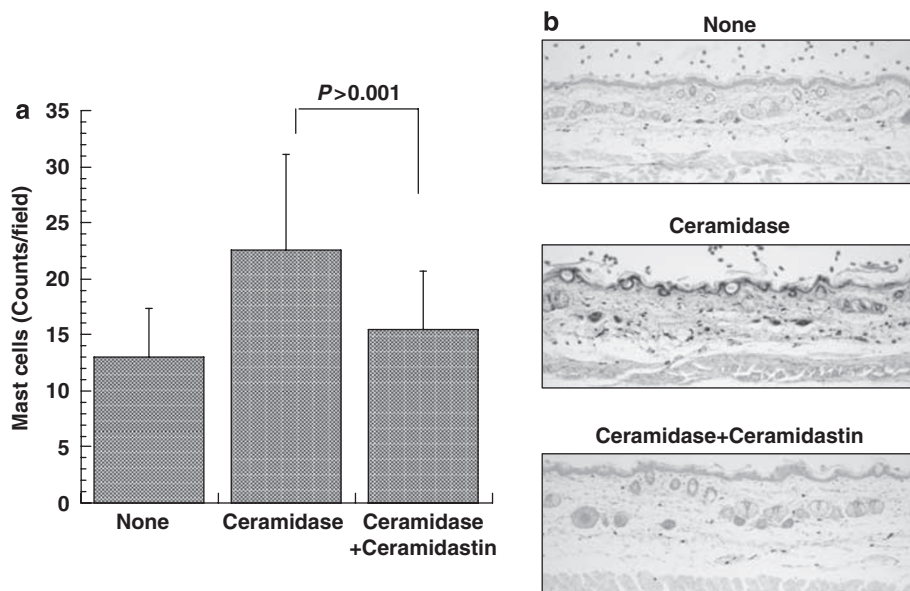Ceramidastin by *Penicillium* sp. Mer-f17067
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Figure 4 Effects of ceramidastin on NC/Nga mice skin. NC/Nga mice (6 mice per group) were treated with *Pseudomonas* ceramidase on the back skin with or without ceramidastin. Mast cells in the dermis were stained by toluidine blue and counted. (**a**) Mast cell counts in the dermis. The values were means ± s.d. of 6 mice. (**b**) Representative photos of the dermis sections stained by toluidine blue.

tions. We used the supernatant of a culture of *P. aeruginosa* no. 12 as a source for bacterial ceramidase. As shown in Figure 3, ceramidastin inhibited *Pseudomonas* ceramidase with an IC_{50} value of $6.25\,\mu g\,ml^{-1}$. On the contrary, ceramidastin did not inhibit human ceramidase even at $200\,\mu g\,ml^{-1}$. Ceramidastin did not show anti-microbial and anti-fungal activities, including against *P. aeruginosa,* at $100\,\mu g\,ml^{-1}$.

We next examined the effect of ceramidastin on atopic dermatitis model NC/Nga mice.[8,9] Mast cells are increased in the dermis with dermatitis.[8] As shown in Figure 4, mast cells were increased in the dermis by *Pseudomonas* ceramidase application. However, ceramidastin treatment significantly inhibited the increase of mast cells in the dermis.

Cytotoxicity *in vitro* and toxicity in mice
Ceramidastin did not show any cytotoxicity against human lung cancer cell lines, HCI-H23 and NCI-H522 cells, human colon cancer HT-29 cells, mouse melanoma B16BL6 cells, mouse colon cancer colon 26 cells, mouse lymphoma cell lines, EL-4 and L-1210 cells up to $100\,\mu g\,ml^{-1}$.

Acute toxicity of ceramidastin in mice was examined using ICR female mice. When ceramidastin was administered intravenously, no fatality was observed up to $6.25\,mg\,kg^{-1}$.

DISCUSSION
It is reported that D-MAPP, a ceramide analog, inhibits human ceramidase.[12] However, ceramidastin did not show inhibitory activity against human ceramidase. It is also reported that GCAS-4 and GCAS-7, other ceramide analogs, as well as D-MAPP inhibit the ceramidase activity of *Pseudomonas* culture with an IC_{50} value of $500\,\mu M$.[13] We tested the effect of D-MAPP on *Pseudomonas* ceramidase in our assay system, and found that D-MAPP inhibited it with an IC_{50} value of $500\,\mu g\,ml^{-1}$. Thus, ceramidastin is considered to be a more selective and stronger inhibitor of bacterial ceramidase.

Ceramidastin is structurally related to rubratoxins.[10] We also examined the effect of rubratoxin A on *Pseudomonas* ceramidase, which showed that rubratoxin A did not inhibit it even at $100\,\mu g\,ml^{-1}$.

Thus, the structural difference of ceramidastin must be significant for its inhibitory action against bacterial ceramidase.

To evaluate the efficacy of ceramidastin on atopic dermatitis, we used NC/Nga mice. It is reported that tacrolimus hydrate improves spontaneous dermatitis in NC/Nga mice.[8] Mast cells are increased in the dermis by allergic responses. Our result showed that ceramidastin inhibited the increase of mast cells in the dermis induced by *Pseudomonas* ceramidase. Ceramidastin showed neither anti-microbial nor anti-fungal activities, which is an advantage because there is a low possibility of inducing drug-resistant bacteria. Thus, it is suggested that ceramidastin would improve atopic dermatitis exacerbated by bacterial infection.

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