ORIGINAL ARTICLE

New verticilides, inhibitors of acyl-CoA:cholesterol acyltransferase, produced by *Verticillium* sp. FKI-2679

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Verticillium sp. FKI-2679, a soil isolate, was found to produce inhibitors of acyl-CoA:cholesterol acyltransferase (ACAT) in a cell-based assay using ACAT1- and ACAT2-expressing CHO cells. Three new compounds, verticilides A2, A3 and B1, were isolated along with a known compound, verticilide A1, from the fermentation broth of the fungus by solvent extraction, ODS column chromatography, silica gel column chromatography and preparative HPLC. Structure elucidation showed that these compounds were new cyclic depsipeptide. Verticilides A1, A2, A3 and B1 showed a degree of selectivity towards ACAT2, with IC₅₀s 8.5–11-fold more potent than observed against ACAT1.

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INTRODUCTION

Acyl-CoA:cholesterol acyltransferase (ACAT, EC 2.3.1.26), an endoplasmic reticulum (ER) membrane protein, is responsible for many functions in the body. ACAT has been postulated as a target for modulation by a new type of anti-atherosclerotic agent.¹ Many pharmaceutical laboratories have developed synthetic ACAT inhibitors; however, none could be successfully developed to date because of either side effects or low *in vivo* efficacy.^{2,3} Recent molecular biological studies have revealed the existence in mammals of two different ACAT isozymes, ACAT1 and ACAT2.^{4–7} ACAT1 is ubiquitously expressed in tissues and cells, whereas ACAT2 is expressed predominantly in the liver (hepatocytes) and intestine.⁸ ACAT1-deficient (*Acat1*^{−/−}) mice are viable and fertile, and ACAT activity is present in the liver and intestine but completely absent in the adrenal cortex and peritoneal macrophages.^{9–11} However, in addition to having dry eye syndrome,⁹ *Acat1*^{−/−} mice lacking apolipoprotein E or the low-density lipoprotein receptor, as atherogenic mouse models, are susceptible to atherosclerosis and had extensive cutaneous xanthomatosis with loss of hair.^{10,11} Free cholesterol content was significantly increased in their skin. In contrast, ACAT activity is completely absent in the liver and intestine of ACAT2-deficient (*Acat2*^{−/−}) mice and these mice are completely resistant to diet-induced hypercholesterolemia and cholesterol gallstone formation.^{12,13} Therefore, it is important to determine the selectivity of inhibitors toward the two ACAT isozymes for their development as new anti-atherosclerotic agents. However, only a few studies have been reported so far.^{14–16}

Before the discovery of the two different ACAT isozymes, our group had focused on the discovery of ACAT inhibitors of microbial origin

in an enzyme assay using rat liver microsomes.¹⁷ Since ACAT isozymes discovery, we refocused on the discovery of ACAT2-selective inhibitors and improved the assay system¹⁵ by utilizing two cell lines, Chinese hamster ovary (CHO) cells expressing African green monkey ACAT1 (ACAT1-CHO cells) and ACAT2 (ACAT2-CHO cells).¹⁴ During our screening programme,^{18,19} four structurally related compounds were isolated from the culture broth of a fungal strain FKI-2679 as inhibitors of both ACAT isozymes in cell-based assays. One was identified as verticilide (verticilide A1 in this study), which had previously been reported to have insecticidal activity,^{20,21} but the others were new compounds, named verticilides A2, A3 and B1 (Figure 1). They are members of the cyclodepsipeptide family such as beauveriolides,^{22–24} beauvericin,²⁵ enniatins²⁶ and bassianolide.²⁷ In this study, the taxonomy of the producing strain, fermentation, isolation, structure elucidation and biological properties of verticilides are described.

RESULTS AND DISCUSSION

Taxonomy of strain FKI-2679

Colonies on potato dextrose agar (PDA) after 7 days at 25 °C (Figure 2a) were 57–60 mm in diameter, floccose to velvety and white (hue number; a). The reverse side was yellowish-gray (hue number; 2ca). Colonies on malt extract agar (MEA) after 7 days at 25 °C (Figure 2b) were 36–38 mm in diameter, floccose to velvety and white (hue number; a). The reverse side was yellowish-gray (hue number; 2ca). Colonies on Miura's medium (LcA) after 7 days at 25 °C (Figure 2c) were 44–45 mm in diameter, floccose to velvety and white (hue number; a). The reverse side was white (hue number; a).

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Figure 1 Structures of verticillides A1, A2, A3 and B1.

Colonies on potato carrot agar (PCA) after 7 days at 25 °C (Figure 2d) were 49–50 mm in diameter, floccose to velvety and white (hue number; a). The reverse side was yellowish-white (hue number; a). Colonies on PDA at 5 and 37 °C were not observed. Conidiophores were from basal hyphae, rarely branching. The phialides were slender, 22.5–47.5 × 1.5–2.5 µm. Conidia were slimy globose heads, subglobose to cylindrical (2.0–4.5 µm). From these morphological characteristics,²⁸ the producing strain FKI-2679 was considered to belong to the genus *Verticillium*. From the taxonomic study, strain FKI-2679 appeared very similar to strain FKI-1033, which was previously reported as a producer of the insecticidal verticillide A1.^{20,21} Though they may be classified to the same species, strain FKI-2679 was isolated from a soil sample collected from Okinawa, Japan, whereas strain FKI-1033 was isolated from a sample collected from Kagoshima, Japan.^{20,21} Furthermore, their secondary metabolites and productivity were different.

Isolation

To the 15-day-old whole broth (10 l) 10 l of ethanol was added. After the mixture was filtered and concentrated to remove ethanol, the residual aqueous solution was extracted with ethyl acetate. The extracts were dried over Na₂SO₄ and concentrated *in vacuo* to dryness to yield a brown oily material (4.4 g). The material was dissolved in hexane–ethyl acetate (20:1, v/v), applied to a silica gel column (130 g)

and eluted stepwise with 20:1, 10:1, 5:1, 3:1 and 1:1 (v/v) of hexane–ethyl acetate and 100:0, 100:1, 50:1, 25:1, 10:1, 1:1 and 0:100 (v/v) of CHCl₃–MeOH (1 l for each solvent). Active fractions from a 1:1 solvent of hexane–ethyl acetate-containing verticillides were concentrated *in vacuo* to dryness to give a red-brown oily material (170 mg). The material was finally purified by preparative HPLC (column: PEGASIL ODS (Senshu Scientific Co. Ltd., Tokyo, Japan), 20 × 250 mm; the solvent is presumably 87% aqueous CH₃CN; detection, UV at 210 nm; flow rate, 8.0 ml min⁻¹). Under these conditions, verticillides B1, A1, A2 and A3 were eluted as peaks with retention times of 19.5, 27.8, 33.1 and 42.8 min, respectively (Figure 3). The fractions were concentrated *in vacuo* to dryness to give pure verticillides A1 (54 mg), A2 (0.91 mg), A3 (0.80 mg) and B1 (4.2 mg) as colorless oils.

Structural elucidation of verticillides A2, A3 and B1

Physico-chemical properties of verticillides A2, A3 and B1. The physico-chemical properties of verticillides A2, A3 and B1 are summarized in Table 1. They had similar UV spectra with end absorption maxima at 202 nm. The IR absorption at 1650–1754 cm⁻¹ suggested the presence of carbonyl groups in their structures. These data were similar to those of verticillide A1,^{20,21} indicating that they were also likely to be cyclodepsipeptides.

Figure 2 Morphological characteristics of verticillide-producing fungal strain FKI-2679. (a) Micrograph of colonies grown on PDA after 7 days. (b) Micrograph of colonies grown on MEA after 7 days. (c) Micrograph colonies grown on PCA after 7 days. (d) Scanning electron micrograph of conidiophores grown on PDA. Scale bar, 10 μ m. (e) Micrograph of synnemata grown on PDA. Scale bar, 10 mm. A full color version of this figure is available at *The Journal of Antibiotics* online.

Figure 3 Chromatographic profile of verticillide purification by preparative HPLC. Column, PEGASIL ODS (20 $\times$ 250 mm); solvent 87% aqueous acetonitrile; detection, UV at 210 nm; flow rate, 8.0 ml min⁻¹.

Amino-acid analysis of verticillides A2, A3 and B1. The acid hydrolysates of verticillides A2, A3 and B1 (6N HCl at 110 $^{\circ}$ C for 24 h) were separated by chiral TLC, and the TLC plates were sprayed with ninhydrin to detect the presence of amino acids by their color

reactions. Only the presence of *N*-methyl-L-alanine was detected for verticillides B1 and A3, whereas both *N*-methyl-L-alanine and L-alanine were found for verticillide A2.

Structure of verticillide A2. The molecular formula of verticillide A2 was determined to be C₄₃H₇₄N₄O₁₂ on the basis of HR-FAB-MS measurement (*m/z*, found 839.5372, calcd 839.5381 for C₄₃H₇₅N₄O₁₂ [M+H]⁺), indicating that it was smaller than verticillide A1 by one carbon atom and two protons. The ¹H and ¹³C NMR spectra (Table 2) resembled those of verticillide A1. The ¹³C NMR spectrum (CDCl₃) showed 43 resolved peaks (Table 2), which were classified into 11 methyl, 16 methylene, 8 methine and 8 carbonyl carbons by analysis of the DEPT spectrum. The connectivity of the proton and carbon atoms was confirmed by the HMQC spectrum (Table 2). As shown by bold lines in Figure 4, eight partial structures contained in the partial structure V were identified by ¹H-¹H COSY. The ¹³C-¹H long-range couplings of ²*J* and ³*J* were measured in the HMBC spectrum (Figure 4a), leading to the following results: (1) the cross peaks from HA- α -H (δ 5.27) and HA- β -H (δ 1.68) to HA-C=O (δ 170.2), from MeAla- β -H (δ 1.42) to MeAla-C=O (δ 171.8), from MeAla- α -H (δ 4.99) to MeAla-C=O and HA-C=O, and from MeAla-NMe-H (δ 3.07) to MeAla- α -C (δ 51.8) and HA-C=O and the ⁴*J* cross peak from MeAla-NMe-H to HA- α -C showed the presence of first 2-hydroxyheptanoyl-*N*-methylalanine moiety (partial structure I); (2) the cross peaks from MeAla- β -H (δ 1.52) to MeAla-C=O (δ 169.9), from MeAla-NMe-H (δ 3.19) to MeAla- α -C (δ 59.5) and HA-C=O (δ 171.2) and the ⁴*J* cross peak from MeAla- α -H (δ 3.75) to HA- α -C (δ 72.0) showed the presence of second 2-hydroxyheptanoyl-*N*-methylalanine moiety (partial structure II); (3) the cross peaks from HA- α -H (δ 5.52) to HA-C=O (δ 169.9), from MeAla- β -H (δ 1.38) to MeAla-C=O (δ 171.9), and from MeAla-NMe-H (δ 2.95) to MeAla- α -C (δ 52.1) and HA-C=O and the ⁴*J* cross peak from MeAla-NMe-H to HA- α -C showed the presence of third 2-hydroxyheptanoyl-*N*-methylalanine

Table 1 Physico-chemical properties of verticilides A1, A2, A3 and B1

	Verticilide A1	Verticilide A2	Verticilide A3	Verticilide B1
Appearance	Colorless oil	Colorless oil	Colorless oil	Colorless oil
$[\alpha]_D^{27}$	-25.0 (c 1.5, CHCl ₃)	-30.7 (c 0.1, CHCl ₃)	-9.48 (c 1.5, CHCl ₃)	-8.48 (c 0.1, CHCl ₃)
Molecular formula	C ₄₄ H ₇₆ N ₄ O ₁₂	C ₄₃ H ₇₄ N ₄ O ₁₂	C ₄₆ H ₈₀ N ₄ O ₁₂	C ₃₃ H ₅₇ N ₃ O ₉
Molecular weight	852	838	880	639
<i>HR-FAB-MS (m/z)</i>				
Calcd	875.5397	839.5381	903.567	662.3993
Found	875.5371 [M+Na] ⁺	839.5372 [M+H] ⁺	903.5651 [M+Na] ⁺	662.4011 [M+Na] ⁺
UV $\lambda_{\max}$ nm in CH ₃ OH	End absorption	End absorption	End absorption	End absorption
IR $\nu_{\max}$ (cm ⁻¹)	2954, 2931, 2871, 2859, 1743, 1664, 1461, 1413, 1195, 1085	2956, 2927, 2871, 2857, 1739, 1660, 1650, 1644, 1455, 1415, 1213, 1089	2954, 2925, 2869, 2854, 1743, 1664, 1461, 1415, 1197, 1085	2956, 2929, 2871, 2859, 1754, 1739, 1664, 1461, 1413, 1344, 1214, 1101
<i>Solubility</i>				
Soluble	CH ₃ OH, CHCl ₃ , EtOAc	CH ₃ OH, CHCl ₃ , EtOAc	CH ₃ OH, CHCl ₃ , EtOAc	CH ₃ OH, CHCl ₃ , EtOAc
Insoluble	H ₂ O	H ₂ O	H ₂ O	H ₂ O

moiety (partial structure III); and (4) the cross peaks from Ala- β -H (δ 1.39) to Ala-C=O (δ 171.9) showed the presence of the alanine moiety (partial structure IV). Furthermore, the long-range couplings from HA- α -H in the partial structure I to MeAla-C=O in the partial structure III, from HA- α -H in the partial structure III to Ala-C=O in the partial structure IV, from HA- α -H in the partial structure V to MeAla-C=O in the partial structure II, and from HA- α -H in the partial structure II to MeAla-C=O in the partial structure I showed the presence of linear octadepsipeptide moiety, as shown in Figure 4b. Considering the remaining atoms (one carbon and one oxygen), the existence of one carbonyl group was supported by its ¹³C NMR chemical shift (δ 171.0). Therefore, it was concluded that the partial structure IV was connected to the partial structure V via an amide bond. From these observations, the structure of verticilide A2 was elucidated as *N*-monodemethyl analog of verticilide A1 (Figure 1) and fulfilled the molecular formula.

Structure of verticilide A3. The molecular formula of verticilide A3 was determined to be C₄₆H₈₀N₄O₁₂ on the basis of HR-FAB-MS measurement (*m/z* found 903.5651, calcd 903.5670 for C₄₆H₈₀N₄O₁₂Na [M+Na]⁺), indicating that it was larger than verticilide A1 by two carbons and four protons, which corresponded to two methylenes from analysis of the DEPT spectra. The ¹H and ¹³C NMR spectra observed in CD₃OD with 1 mM potassium thiocyanate (Table 2) resembled those of verticilide A1 observed in CD₃OD with 300 mM potassium thiocyanate. From the detailed analysis of the ¹H-¹H COSY and the ¹H-¹³C HMBC experiments, verticilide A3 was found to be composed of 2-hydroxyheptanoyl-*N*-methylalanine and 2-hydroxynonanoyl-*N*-methylalanine moieties in a ratio of 3:1. Furthermore, fragment ion peaks were observed at *m/z* 242, 370, 455, 583, 668, 796 and 881 in FAB-MS (Figure 5). From these observations, the structure of verticilide A3 was elucidated as shown in Figure 1 and fulfilled the molecular formula.

Structure of verticilide B1. The molecular formula of verticilide B1 was determined to be C₃₃H₅₇N₃O₉ on the basis of HR-FAB-MS measurement (*m/z* found 662.4011, calcd 662.3993 for C₃₃H₅₇N₃

O₉Na [M+Na]⁺). The ¹³C NMR spectrum (CDCl₃) showed 11 resolved peaks (Table 2), which were classified into three methyl, four methylene, two methine and two carbonyl carbons by analysis of the DEPT spectrum. The ¹H NMR spectrum in CDCl₃ displayed nine proton signals (Table 2). These data suggested that verticilide B1 was a cyclic trimer composed of C₁₁H₁₉NO₃ units. The connectivity of proton and carbon atoms was confirmed by the HMQC spectrum (Table 2). As shown by bold lines in Figure 6, the three partial structures were elucidated by ¹H-¹H COSY. The ¹³C-¹H long-range couplings of ²*J* and ³*J* in the HMBC spectrum are also shown in Figure 6, leading to the following results: (1) the cross peaks from HA- β -H (δ 1.78) to HA- δ -C (δ 32.5), from HA- ζ -H (δ 0.92) to HA- δ -C, and from HA- β -H to HA-C=O (δ 167.5) showed the presence of a 2-hydroxyheptanoic acid moiety; (2) The long-range couplings from MeAla-NMe-H (δ 3.08) to MeAla- α -C (δ 53.5), from MeAla- α -H (δ 5.06) to MeAla-C=O (δ 172.2), and from MeAla- β -H (δ 1.42) to MeAla-C=O showed the presence of an *N*-methylalanine moiety, supporting the result of amino-acid hydrolysis of verticilide B1. Thus, the C₁₁H₁₉NO₃ units of verticilide B1 (partial structure I) was elucidated as shown in Figure 6. Furthermore, the long-range couplings from MeAla-NMe-H and MeAla- α -H to HA-C=O showed that 2-hydroxypentanoic acid was attached to *N*-methylalanine via an amide bond. Therefore, verticilide B1 was a cyclic trimer constructed with partial structure I. From these observations, the structure of verticilide B1 was elucidated as shown in Figure 1 and fulfilled the molecular formula.

Inhibition of cholesteryl ester (CE) synthesis in ACAT1- and ACAT2-CHO cells

The effect of verticilides on ACAT1 and ACAT2 isozymes was evaluated in a cell-based assay using ACAT1- and ACAT2-CHO cells. As shown by the IC₅₀ values summarized in Table 3, all of the verticilides inhibited both ACAT1 and ACAT2; they showed, however, a degree of selective inhibition (more potent IC₅₀s) towards ACAT2 compared with ACAT1, with selectivity index (SI) of 8.5–11. A number of synthetic ACAT inhibitors have been reported, but no

Table 2 ^1H and ^{13}C NMR chemical shifts of verticilides A2, A3 and B1

	δ_{H} (p.p.m.) ^a	δ_{C} (p.p.m.) ^b		δ_{H} (p.p.m.) ^a	δ_{C} (p.p.m.) ^b
Verticilide A2 (in CDCl_3)					
(Unit I)			(Unit III)		
N-methylalanine (MeAla)			N-methylalanine (MeAla)		
C=O	—	171.8 (s)	C=O	—	171.9 (s)
α	4.99 (1H, m)	51.8 (d)	α	5.35 (1H, m)	52.1 (d)
β	1.42 (3H, d, $J=7.2$)	14.3 (q)	β	1.38 (3H, d, $J=7.2$)	14.5 (q)
NCH_3	3.07 (3H, s)	30.3 (q)	NCH_3	2.95 (3H, s)	31.2 (q)
2-Hydroxyheptanoic acid (HA)			2-Hydroxyheptanoic acid (HA)		
C=O	—	170.2 (s)	C=O	—	169.9 (s)
α	5.27 (1H, t, $J=7.2$)	71.2 (d)	α	5.52 (1H, m)	69.9 (d)
β	1.68 (2H, m)	30.7 (t)	β	1.82 (2H, m)	31.4 (t)
γ } δ } ϵ }	1.20~1.50 (2H, m)	22.5~32.0 ^c (t)	γ } δ } ϵ }	1.20~1.50 (2H, m)	22.5~32.0 ^c (t)
ζ	0.88 (3H, m)	13.9~14.4 ^d (q)	ζ	0.88 (3H, m)	13.9~14.4 ^d (q)
(Unit II)			(Unit IV)		
N-methylalanine (MeAla)			Alanine (Ala)		
C=O	—	169.9 (s)	C=O	—	171.9 (s)
α	3.75 (1H, m)	59.5 (d)	α	4.71 (1H, t, $J=8.0$)	47.1 (d)
β	1.52 (3H, d, $J=7.2$)	13.5 (q)	β	1.39 (3H, d, $J=7.2$)	15.8 (q)
NCH_3	3.19 (3H, s)	36.8 (q)	NH	7.45 (3H, bs)	—
2-Hydroxyheptanoic acid (HA)			(Unit V)		
C=O	—	171.2 (s)	2-Hydroxyheptanoic acid (HA)		
α	5.00 (1H, m)	72.0 (d)	C=O	—	170.0 (s)
β	1.58 (2H, m)	24.8 (t)	α	4.89 (1H, m)	75.9 (d)
γ } δ } ϵ }	1.20~1.50 (2H, m)	22.5~32.0 ^c (t)	β	1.75 (2H, m)	31.0 (t)
ζ	0.88 (3H, m)	13.9~14.4 ^d (q)	γ } δ } ϵ }	1.20~1.50 (2H, m)	22.5~32.0 ^c (t)
			ζ	0.88 (3H, m)	13.9~14.4 ^d (q)
Verticilide A3 (in CD_3OD with 1 mM KSCN)					
(Unit I)			(Unit II)		
N-methylalanine (MeAla)			N-methylalanine (MeAla)		
C=O	—	173.0 (s)	C=O	—	173.0 (s)
α	5.36 (1H, br.s)	53.9 (d)	α	5.36 (1H, br.s)	53.9 (d)
β	1.48 (3H, d, $J=7.2$)	14.6 (q)	β	1.48 (3H, d, $J=7.2$)	14.6 (q)
NCH_3	3.07 (3H, s)	31.9 (q)	NCH_3	3.07 (3H, s)	31.9 (q)
2-Hydroxyheptanoic acid (HA)			2-Hydroxynonanoic acid (HA)		
C=O	—	172.3 (s)	C=O	—	172.3 (s)
α	5.30 (1H, t, $J=6.4$)	73.8 (d)	α	5.30 (1H, t, $J=6.4$)	73.8 (d)
β	1.82 (2H, m)	31.5 (t)	β	1.82 (2H, m)	31.5 (t)
γ	1.52 (2H, m)	25.8 (t)	γ }	1.52 (2H, m)	26.1 (t)
δ	1.38 (2H, m)	32.5 (t)	δ }	1.36 (2H)	30.1, 30.2 (t)
ϵ	1.38 (2H, m)	23.4 (t)	ϵ }		
ζ	0.94 (3H, m)	14.2 (q)	ζ	1.38 (2H, m)	32.8 (t)
			η	1.38 (2H, m)	23.6 (t)
			θ	0.94 (3H, m)	14.4 (q)
Verticilide B1 (in CDCl_3)					
N-methylalanine (MeAla)					
C=O	—	172.2 (s)			
α	5.06 (1H, q, $J=7.6$)	53.5 (d)			
β	1.42 (3H, d, $J=7.2$)	14.5 (q)			
NCH_3	3.08 (3H, s)	31.5 (q)			

Table 2 (Continued)

	δ_H (p.p.m.) ^a	δ_C (p.p.m.) ^b	δ_H (p.p.m.) ^a	δ_C (p.p.m.) ^b
2-Hydroxyheptanoic acid (HA)				
C=O	—	167.5 (s)		
α	5.36 (1H, dd, $J=6.1, 7.6$)	72.4 (d)		
β	1.78 (2H, m)	31.8 (t)		
γ	1.34~1.50 (2H, m)	26.0 (t)		
δ	1.34 (2H, m)	32.5 (t)		
ϵ	1.34 (2H, m)	23.5 (t)		
ζ	0.92 (3H, t, $J=6.4$)	14.3 (q)		

^aSpectra taken at 400 MHz.^bSpectra taken at 100 MHz.^c22.4, 22.4, 24.8, 25.3, 29.5, 29.7, 30.4, 31.0, 31.3, 31.3, 32.0, 34.0 p.p.m.^d13.9, 14.0, 14.1, 14.4 p.p.m. **Figure 4** Partial structures I to V (a) and ¹³C-¹H HMBC and ROESY experiments (b) of verticilide A2.

inhibitors have been clinically developed to date. Recently, attempts to use pactimibe²⁹ and avasimibe,^{30,31} which were reported to inhibit both ACAT1 and ACAT2, clinically failed. On the other hand, our research group has been interested in ACAT2-selective inhibitors such

Figure 5 Mass fragments analysis of verticilide A3 observed from FAB mass spectrum.

as the pyripyropenes as a different approach for the development of new anti-atherosclerotic agents.³² In this sense, verticilides might represent potential leads beyond the pyripyropenes, although their ACAT2 selectivity is insufficient. We previously reported that two types of depsipeptides, beauveriolides and beauvericin, had ACAT inhibitory activity^{25,33} and investigated their selectivity toward the two isozymes. Beauveriolides I and III were more selective toward ACAT1, while beauvericin inhibited both ACAT1 and ACAT2.¹⁵ Thus, these depsipeptides including the verticilides showed ACAT inhibitory properties but their isozyme selectivity appears diverse.

Other biological activities

Verticilide A1 was reported to inhibit the binding between ryanodine (insecticidal plant alkaloid) and cockroach ryanodine receptor with an IC₅₀ value of 4.2 μM.^{20,21} Verticilides A2, A3 and B1 did not, however, inhibit this binding event at concentrations up to 100 μM. Verticilide B1 had the broad antimicrobial activity; active against *Bacillus subtilis*,

Figure 6 Partial structure I of verticilide B1.

Table 3 Effect of verticilides on ACAT1 and ACAT2 activity in ACAT1- and ACAT2-CHO cells

Verticilide	IC ₅₀ (μM)		S ^a
	ACAT1	ACAT2	
A1	2.5	0.23	11
A2	4.8	0.55	8.7
A3	3.5	0.36	9.7
B1	11	1.3	8.5

^aSelectivity index (SI); IC₅₀ for ACAT1/IC₅₀ for ACAT2.

Staphylococcus aureus, *Micrococcus luteus*, *Pseudomonas aeruginosa* and *Mucor racemosus*. On the other hand, verticilides A1 and A2 had limited antimicrobial activity; active against *P. aeruginosa* and *M. racemosus*, and against *P. aeruginosa*, respectively. Verticilide A3 showed no antimicrobial activity. Verticilides A1, A3 and B1 also showed moderate nematocidal activity against *Artemia salina* (data not shown).

METHODS

General experimental procedures

Fungal strain FKI-2679 was originally isolated from a soil sample collected on Ishigaki Island, Okinawa, Japan. This strain was used for the production of verticilides A1, A2, A3 and B1. Kiesegel 60 (Merck KGaA, Darmstadt, Germany) was used for silica gel column chromatography. To determine the amounts of verticilides A1, A2, A3 and B1 in culture broths, samples (ethyl acetate extracts) dissolved in methanol were analyzed using a HP1100 system (Hewlett-Packard Company, Palo Alto, CA, USA) under the following conditions: column, symmetry (2.1×150 mm; Waters Corporation, Milford, MA, USA); flow rate, 0.2 ml min⁻¹; mobile phase, a 20-min linear gradient from 60% aqueous MeCN to 100% MeCN containing 0.05% H₃PO₄; detection, UV at 210 nm.

UV spectra were recorded on a spectrophotometer (8453 UV-Visible spectrophotometer; Agilent Technologies Inc., Santa Clara, CA, USA). IR spectra were recorded on a Fourier transform infrared spectrometer (FT-710; Horiba Ltd., Kyoto, Japan). Optical rotations were measured with a digital polarimeter (DIP-1000; JASCO Co., Tokyo, Japan). FAB-MS spectra were recorded on a mass spectrometer (JMS-DX300; JEOL Ltd., Tokyo, Japan). Various NMR spectra were measured with a spectrometer (XL-400; Varian Inc., Palo Alto, CA, USA).

Taxonomic studies of the producing strain FKI-2679

Fungal strain FKI-2679 was isolated from soil collected on Ishigaki Island, Okinawa, Japan. For determination of the morphological characteristics, the

isolate was inoculated as a 1-point culture on PDA, MEA, LcA and PCA, and grown for 7 days at 25 °C (also at 5 and 37 °C on PDA) in the dark. Color Harmony Manual fourth Edition (Container Corporation of America, Chicago, IL, USA) was used to determine color names and hue numbers.³⁴ For the determination of micro-morphological characteristics, the samples were observed under a Vanox-S AH-2 microscope (Olympus, Tokyo, Japan) and JSM-5600 scanning electron microscope (JEOL, Tokyo, Japan). The D1/D2 rDNA sequence of the strain was deposited at the DDBJ with accession number AB540177.

Fermentation

A slant culture of strain FKI-2679 grown on LCA (glycerol 0.10%, KH₂PO₄ 0.080%, K₂HPO₄ 0.020%, MgSO₄·7H₂O 0.020%, KCl 0.020%, NaNO₃ 0.20%, yeast extract 0.020%, agar 1.5%, pH 6.0) was inoculated into a 50-ml tube containing 10 ml seed medium (glucose 2.0%, polypeptone 0.50%, MgSO₄·7H₂O 0.050%, yeast extract 0.20%, KH₂PO₄ 0.10%, agar 0.10%, pH 6.0). The tube was shaken on a reciprocal shaker for 2 days at 27 °C. A 1-ml portion of the seed culture was then inoculated into a 500-ml Erlenmeyer flask containing the production medium (50 g Italian rice; Japan Europe Trading Co. Ltd., Tokyo, Japan). Fermentation was carried out at 27 °C for 6 days under stable conditions.

Acid hydrolysis

Verticilides A2, A3 and B1 (100 μg each) were degraded in the gas phase of 6 N HCl (200 μl) at 110 °C for 24 h using the PICO-TAG work station (Waters Corporation).²⁰ To determine the stereochemistry of alanine and *N*-methyl-L-alanine, the degradation products were dissolved in MeOH (100 μl) and separated on an HPTLC plate (chiral plate, CHIR; Merck, Darmstadt, Germany) with the solvent system 0.05 M KH₂PO₄/MeOH/MeCN=5:5:1 and amino acids were detected by ninhydrin reagent.

Cell culture

Two cell lines, CHO cells expressing ACAT1 and ACAT2 isozymes of African green monkey (ACAT1- and ACAT2-CHO cells, respectively)¹⁴ were kind gifts from Dr LL Rudel (Wake Forest University, Winston Salem, NC, USA). Cells were maintained as described previously.¹⁵

Assay for ACAT1 and ACAT2 activities using ACAT1- and ACAT2-CHO cells

Assays for ACAT1 and ACAT2 activities using ACAT1- and ACAT2-CHO cells were carried out by our established method.¹⁵ Briefly, ACAT1- or ACAT2-CHO cells (1.25×10⁵ 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% CO₂. The assays were done with cells that were at least 80% confluent. Following the overnight recovery, test sample (2.5 μl MeOH solution) and [1-¹⁴C]oleic acid (5 μl 10% EtOH/PBS solution, 1 nmol, 1.85 kBq) were added to each culture. After a 6-h incubation at 37 °C in 5% CO₂, the medium was removed and the cells in each well were washed twice with PBS. 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 [¹⁴C]CE was analyzed with a BAS2000 analyzer (Fuji Film, Tokyo, Japan). In this cell-based assay, [¹⁴C]CE was produced by the reaction of ACAT1 or ACAT2. ACAT inhibitory activity (%) is defined as (1-[¹⁴C]CE-drug/[¹⁴C]CE-control)×100. The IC₅₀ value is defined as the drug concentration causing 50% inhibition of a biological activity.

Other biological assays

Antimicrobial activity against 14 species of microorganisms was measured by a paper disk method. Media for growth of microorganisms were as follows: GAM agar (Nissui Seiyaku Co., Tokyo, Japan) for *Bacteroides fragilis*; Waksman agar for *Mycobacterium smegmatis*; Bacto PPLO agar (Difco) supplemented with 15% horse serum, 0.1% glucose, 0.2% phenol red (5 mg ml⁻¹) and 1.5% agar for *Acholeplasma laidlawii*; nutrient agar for *B. subtilis*, *S. aureus*, *M. luteus*, *Escherichia coli*, *P. aeruginosa* and *Xanthomonas oryzae*; a medium composed of 1.0% glucose, 0.5% yeast extract and 0.8% agar for *Pyricularia oryzae*, *Aspergillus niger*,

M. racemosus, *Candida albicans* and *Saccharomyces cerevisiae*. A paper disk (i.d. 6 mm, Advantec) containing 10 µg of a sample was placed on the agar plate. Bacteria except *X. oryzae* were incubated at 37 °C for 24 h. Yeasts and *X. oryzae* were incubated at 27 °C for 24 h. Fungi were incubated at 27 °C for 48 h. The insecticidal assay used *Artemia salina* as test organism. Briefly, about 10 nuclei larvae hatched from eggs of *A. salina* in the culture medium (295 µl, 0.24% Tris, 2.57% NaCl, 0.47% MgCl₂, 0.07% KCl, 0.02% Na₂CO₃, 0.64% MgSO₄ and 0.11% CaCl₂, pH 7.0) were incubated with a sample (5 µl MeOH solution) in a well of a 96-well microplate at 20 °C. After 48 h, the motility of *A. salina* was assessed visually and compared with the control (no test sample).

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