

## ORIGINAL ARTICLE

# Verticilide, a new ryanodine-binding inhibitor, produced by *Verticillium* sp. FKI-1033

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**A new ryanodine-binding inhibitor, verticilide, was isolated from the cultured broth of a fungus, *Verticillium* sp. FKI-1033. It is a 24-membered ring cyclic depsipeptide, its structure being elucidated as cyclo[(2*R*)-2-hydroxyheptanoyl-*N*-methyl-L-alanyl]<sub>4</sub>. Verticilide inhibited ryanodine binding to ryanodine receptors in the cockroach at an IC<sub>50</sub> value of 4.2 μM, whereas inhibition against mouse ryanodine receptors was weak (IC<sub>50</sub>=53.9 μM).**

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**Keywords:** depsipeptide; insecticide; ryanodine-binding inhibitor; ryanodine receptor; verticilide; *Verticillium*

## INTRODUCTION

In the course of screening for insecticidal compounds, we have isolated some new antibiotics from microbial metabolites.<sup>1</sup> Recently, our attention turned to ryanodine receptors as a potential target for new insecticides.

Ryanodine was first isolated as an insecticide from the stem and roots of the plant *Ryania speciosa*. It was shown to bind Ca<sup>2+</sup> channel of the sarcoplasmic reticulum, leading to the discovery of ryanodine receptors. A ryanodine receptor (RyR) is a Ca<sup>2+</sup> release channel found in the intracellular sarcoplasmic or endoplasmic reticulum of a variety of cells.<sup>2,3</sup> There are three different isoforms (RyR1, RyR2 and RyR3) found in mammals. RyR1 is abundant in the skeletal muscle, RyR2 is abundant in the cardiac muscle and RyR3 is found in the brain and also at lower levels in a variety of tissues. RyRs control intracellular Ca<sup>2+</sup> release in skeletal and cardiac muscles, activating muscle contraction during excitation–contraction (EC) coupling. In skeletal muscle, EC coupling occurs through voltage-gated Ca<sup>2+</sup> release, controlled by direct protein–protein interactions between L-type Ca<sup>2+</sup> channel at the plasma membrane and RyR1. Conversely, RyR2 and RyR3 are activated by elevated Ca<sup>2+</sup> concentrations through the L-type Ca<sup>2+</sup> channel, which is called Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release.

Dantrolene is a drug used for malignant hyperthermia, which is a pharmacogenetic disorder triggered by halogenated anesthetics, and is typically inhaled during surgery. Dantrolene is believed to inhibit RyR1 and RyR3 but not RyR2.<sup>4</sup> The RyRs are potential therapeutic targets for diverse human disorders of cardiac and skeletal muscles and of the central nervous system.

In contrast to mammalian receptors, insects have a single but distinct RyR.<sup>5</sup> Therefore, insect RyR is a potential target for new

insecticides.<sup>6</sup> The molecular target of some new synthetic insecticides, flubendiamide and anthranilic diamide, was shown to be RyR.<sup>7,8</sup> Consequently, we screened for ryanodine-binding inhibitors specific to insect RyR from microbial origins and isolated a new compound, verticilide (**1**, Figure 1), produced by a cultured broth of *Verticillium* sp. FKI-1033.<sup>9</sup>

We have already reported the stereochemical study and the total synthesis of **1**.<sup>10</sup> In this report, we describe the taxonomy of the producing strain, the fermentation, isolation and the planar structure elucidation with detailed NMR assignments of **1** and its ryanodine-binding inhibitory activity compared with the activity of the structure-related compounds. The other biological activities of **1** are also described.

## RESULTS AND DISCUSSION

### Taxonomy of the producing organism

Strain FKI-1033 was originally isolated from a soil sample collected in Kagoshima Prefecture, Japan. Colonies of strain FKI-1033 grown on potato dextrose agar were 42–44 mm in diameter after 7 days at 25 °C, floccose to velvety and white in color. The reverse side was white to pale-yellowish brown. Microscopic observation of colonies grown on malt extract agar identified hyphae with septa, with conidiophores born from the aerial mycelia, with occasional branching (Figure 2). The phialide was observed directly from aerial hyphae, or alone or two to four verticillations in the middle part or top of conidiophores. The phialide was 25–80 μm with slight swelling at the base (2.0–2.8 μm) and narrowing into a conical shape at the top. Conidia were sugulose to oval (2.5–4.0 × 2.0–3.0 μm) and formed a viscous conidial mass at the top of the phialide.

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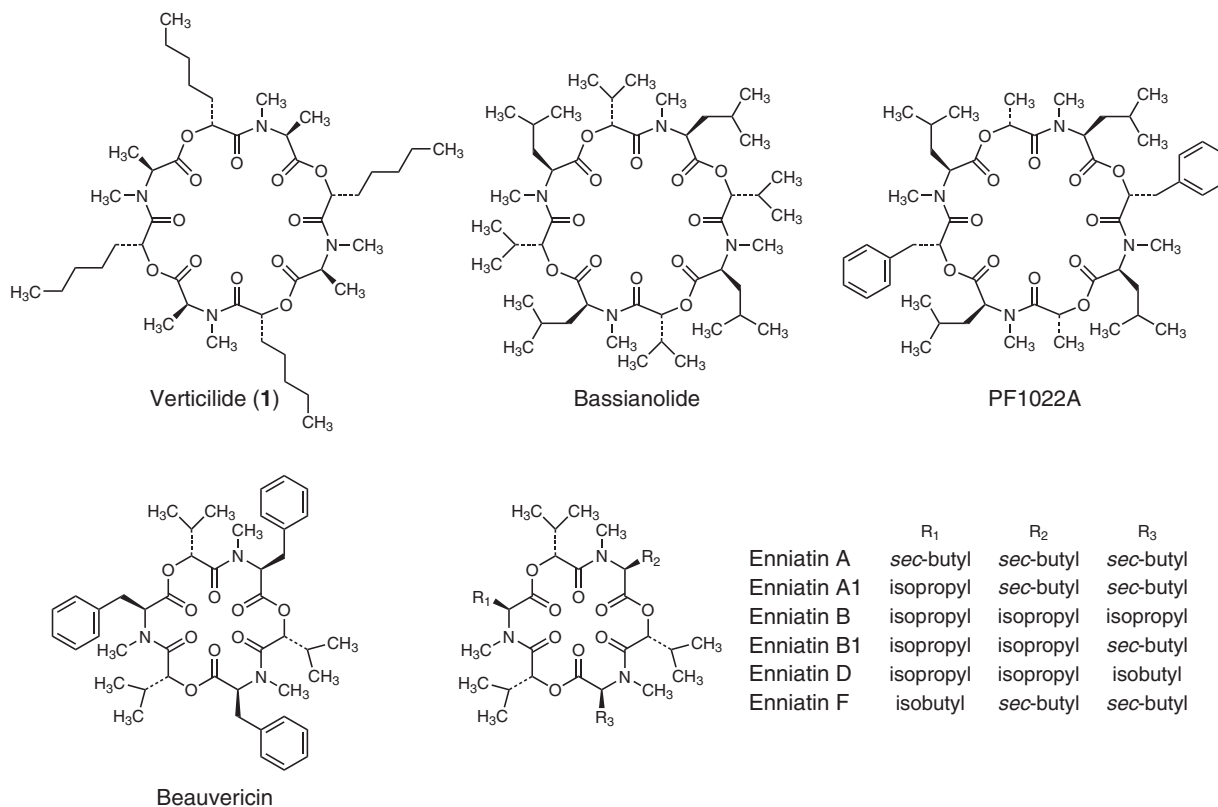


Figure 1 Structures of verticilide (1) and the other cyclic depsipeptides.

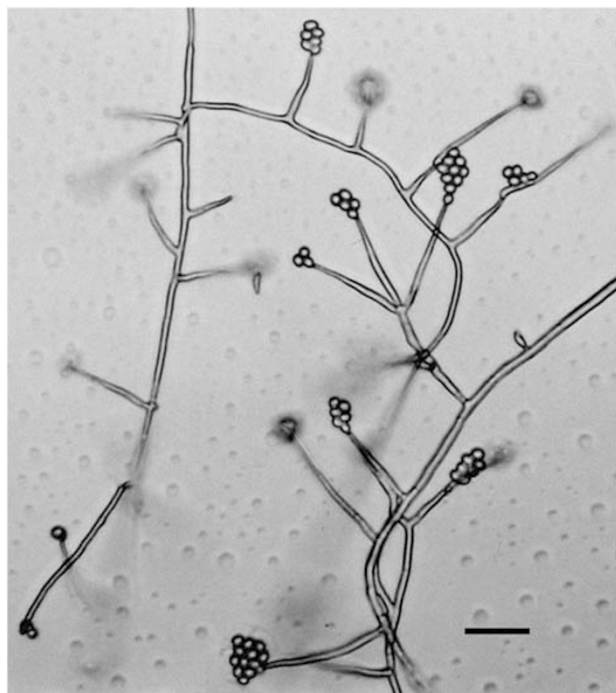


Figure 2 Photomicrograph of strain FKI-1033. Bar=10  $\mu$ m.

From the above characteristics, the microorganism was considered to belong to the genus *Verticillium* Nees<sup>11</sup> and was named *Verticillium* sp. FKI-1033. The strain was deposited at the International Patent

Organism Depository, National Institute of Advanced Industrial Science and Technology, Tsukuba, Japan, as FERM BP-8219.

#### Fermentation

A stock culture of strain FKI-1033 was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of a seed medium and incubated on a rotary shaker at 27 °C for 3 days. The seed broth was diluted to five times with sterilized water and 10 ml of the diluted broth was transferred into each of twenty-four 1-l Roux flasks containing 240 g of a production medium. Fermentation was carried out statically at 27 °C for 13 days.

#### Isolation

After 300 ml of methanol-H<sub>2</sub>O (2:1) was added to each flask, they were vigorously shaken and then kept for 3 h. The extract solutions in each flask were combined, the methanol in the solution was removed by evaporation and the remaining water solution was partitioned with ethyl acetate twice, the organic layer finally being concentrated *in vacuo* to afford a brown oil (2.53 g). This was applied to a silica gel column ( $\phi$  2.8 $\times$ 14 cm, Silica gel 60, Merck 107734, Merck KGaA, Darmstadt, Germany) and washed with hexane-ethyl acetate (4:3). Active fractions eluted with hexane-ethyl acetate (2:3) were concentrated to yield a brown oil (183 mg), which was applied to a Sephadex LH-20 column (GE Healthcare UK Ltd., Little Chalfont, UK) ( $\phi$  2.7 $\times$ 92 cm) and eluted with methanol to afford a colorless oil of **1** (86.5 mg).

#### Structure elucidation

The physico-chemical properties of **1** are summarized in Table 1. The molecular formula of **1** was established as C<sub>44</sub>H<sub>76</sub>N<sub>4</sub>O<sub>12</sub> using

HR-FAB-MS. The IR absorbances observed at 1743 and 1662  $\text{cm}^{-1}$  suggested the presence of ester carbonyl groups and amide carbonyl groups, respectively. The  $^1\text{H}$  and  $^{13}\text{C}$  NMR of **1** showed very

complicated spectra. Their chemical shifts are shown in Table 2. The signals of  $\delta_{\text{H}}$  4.5–5.6 and  $\delta_{\text{C}}$  51–55 suggested  $\alpha$ -carbons of amino acids and the signals of  $\delta_{\text{C}}$  169–172 and the IR absorbances (see above) suggested ester carbonyl and amide carbonyl carbons.

The initial structure elucidation of **1** was thus carried out using its acid hydrolysis, which afforded *N*-methyl-L-alanine (**2**) and (2*R*)-2-hydroxyheptanoic acid (**3**) as reported previously.<sup>10</sup> Mild alkaline hydrolysis (0.24 M sodium hydroxide for 24 h) of **1** afforded **4**, which was elucidated as 2-hydroxyheptanoyl-*N*-methylalanine using spectroscopic data.

The molecular formula of **1** suggested that it is a cyclic tetramer of **4**, and the  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of **1** are similar to those of **4**. However, > 50 carbon signals were observed in the  $^{13}\text{C}$  NMR of **1**, which suggests that it exists in two conformers. Detailed analysis, using COSY and heteronuclear multiple bond correlation experiments of **1**, revealed that the ratio of conformer A (asymmetric conformer) and conformer B (symmetric conformer) is 3:4 (Table 2 and Figure 3). Similar conformer mixtures have been reported for symmetric 24-membered ring depsipeptides, bassianolide and PF1022A.<sup>12,13</sup> NMR spectra of PF1022A are known to be simplified by addition of potassium thiocyanate (M Ohyama, personal communication). When the NMR spectra were observed in  $\text{CD}_3\text{OD}$  with potassium thiocyanate (30 mM of **1** and 300 mM of potassium thiocyanate), the spectra showed only one symmetric conformer (Table 2). Thus, the

**Table 1** Physico-chemical properties of verticilide (**1**)

Appearance	Colorless oil
$[\alpha]_{\text{D}}^{25}$	-53.0° (c 0.2, MeOH)
Molecular formula	$\text{C}_{44}\text{H}_{76}\text{N}_4\text{O}_{12}$
Molecular weight	853.1
HR-FAB-MS ( <i>m/z</i> )	
Found	875.5397 (M+Na) <sup>+</sup>
Calcd	875.5357 for $\text{C}_{44}\text{H}_{76}\text{N}_4\text{O}_{12}\text{Na}$
UV $\lambda_{\text{max}}^{\text{MeOH}}$ nm ( $\epsilon$ )	202 (19700)
IR $\nu_{\text{max}}$ (KBr) $\text{cm}^{-1}$	3467, 2956, 2933, 2862, 1743, 1662, 1464, 1416, 1205, 1084
Solubility	
Soluble	MeOH, EtOH, EtOAc, $\text{CHCl}_3$
Insoluble	$\text{H}_2\text{O}$ , <i>n</i> -hexane
Color reaction	
Positive	$\text{H}_2\text{SO}_4$

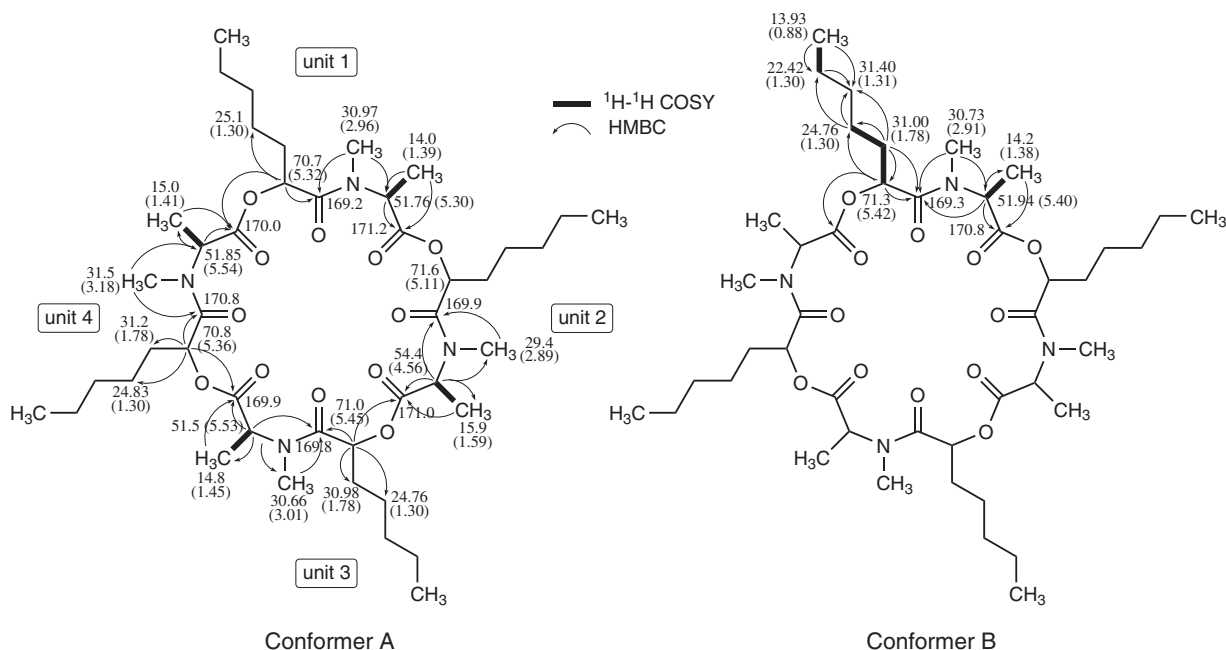
**Table 2** NMR data of verticilide (**1**)

	Conformer A, unit 1 <sup>a</sup>		Conformer A, unit 2 <sup>a</sup>		Conformer A, unit 3 <sup>a</sup>	
	$\delta_{\text{C}}$	$\delta_{\text{H}}$	$\delta_{\text{C}}$	$\delta_{\text{H}}$	$\delta_{\text{C}}$	$\delta_{\text{H}}$
<b>2-Hydroxyheptanoic acid</b>						
$\alpha$	70.7 d	5.32 dd (1H, $J=3.3, 10.1$ Hz)	71.6 d	5.11 dd (1H, $J=2.5, 10.6$ Hz)	71.0 d	5.45 dd (1H, $J=5.3, 8.4$ Hz)
$\beta$	31.1 <sup>b</sup> t	1.78 m (2H)	30.92 <sup>b</sup> t	1.78 m (2H)	30.98 t	1.78 m (2H)
$\gamma$	25.1 t	1.30 m (2H)	24.82 t	1.30 m (2H)	24.76 t	1.30 m (2H)
$\delta$	31.40 <sup>c</sup> t	1.31 m (2H)	31.40 <sup>c</sup> t	1.31 m (2H)	31.36 <sup>c</sup> t	1.31 m (2H)
$\epsilon$	22.42 <sup>d</sup> t	1.30 m (2H)	22.39 <sup>d</sup> t	1.30 m (2H)	22.39 <sup>d</sup> t	1.30 m (2H)
$\zeta$	13.90 <sup>e</sup> q	0.88 m (3H)	13.88 <sup>e</sup> q	0.88 m (3H)	13.88 <sup>e</sup> q	0.88 m (3H)
C=O	169.2 s		169.9 s		169.8 s	
<b>N-Methylalanine</b>						
NMe	30.97 q	2.96 s (3H)	29.4 q	2.89 s (3H)	30.66 q	3.01 s (3H)
$\alpha$	51.76 d	5.30 q (1H, $J=7.4$ Hz)	54.4 d	4.56 q (1H, $J=7.3$ Hz)	51.5 d	5.53 q (1H, $J=7.3$ Hz)
$\beta$	14.0 q	1.39 d (3H, $J=7.4$ Hz)	15.9 q	1.59 d (3H, $J=7.3$ Hz)	14.8 q	1.45 d (3H, $J=7.3$ Hz)
C=O	171.2 s		171.0 s		169.9 s	
<b>Conformer A, unit 4<sup>a</sup></b>						
<b>2-Hydroxyheptanoic acid</b>						
$\alpha$	70.8 d	5.36 dd (1H, $J=5.3, 9.0$ Hz)	71.3 d	5.42 m (3H)	73.9 d	5.32 dd (1H, $J=4.1, 8.2$ Hz)
$\beta$	31.2 t	1.78 m (2H)	31.00 t	1.78 m (6H)	31.6 t	1.82 m (2H)
$\gamma$	24.83 t	1.30 m (2H)	24.76 t	1.30 m (6H)	25.8 t	1.52 m (2H)
$\delta$	31.36 <sup>c</sup> t	1.31 m (2H)	31.40 t	1.31 m (6H)	32.4 t	1.37 m (2H)
$\epsilon$	22.39 <sup>d</sup> t	1.30 m (2H)	22.42 t	1.30 m (6H)	23.4 t	1.37 m (2H)
$\zeta$	13.88 <sup>e</sup> q	0.88 m (3H)	13.93 q	0.88 m (9H)	14.1 q	0.93 t (3H, $J=6.2$ Hz)
C=O	170.8 s		169.3 s		172.3 s	
<b>Conformer B<sup>a</sup></b>						
<b>N-Methylalanine</b>						
NMe	31.5 q	3.18 s (3H)	30.73 q	2.91 s (9H)	32.0 q	3.07 s (3H)
$\alpha$	51.85 d	5.54 q (1H, $J=7.4$ Hz)	51.94 d	5.40 q (3H, $J=7.2$ Hz)	54.2 d	5.39 br.s (1H)
$\beta$	15.0 q	1.41 d (3H, $J=7.4$ Hz)	14.2 q	1.38 d (9H, $J=7.2$ Hz)	14.6 q	1.50 d (3H, $J=7.1$ Hz)
C=O	170.0 s		170.8 s		173.1 s	

Abbreviations: d, doublet; H, number of proton; J, coupling constant; m, multiplet; q, quartet; s, singlet; t, triplet.

<sup>a</sup>The spectra of conformers A (units 1–4) and B mixture were measured in  $\text{CDCl}_3$ .

<sup>b–e</sup>Assignment could be interchanged.



**Figure 3**  $^1\text{H}$ - $^1\text{H}$  COSY and heteronuclear multiple bond correlation (HMBC) correlations of verticilide (**1**). The numbers are  $^{13}\text{C}$  chemical shifts and  $^1\text{H}$  chemical shifts (in parentheses).

structure of **1** was elucidated as cyclo[(2*R*)-2-hydroxyheptanoyl-*N*-methyl-*L*-alanyl] $_4$  (Figure 1). This was confirmed using total synthesis.<sup>10</sup>

Although microorganisms produce various cyclic depsipeptides, there are few reports of natural 24-membered ring depsipeptides that contain alternate arrangements of amino and hydroxy acids. The antifungal amidomycin, cyclo[(2*R*)-2-hydroxy-3-methylbutanoyl-*D*-valyl] $_4$ , and cytotoxic montanastatin, cyclo[(2*R*)-2-hydroxy-3-methylbutanoyl-*D*-valyl-(2*S*)-2-hydroxypropanoyl-*L*-valyl] $_2$ , are produced by *Streptomyces* spp.<sup>14,15</sup> As for fungi, bassianolide, cyclo[(2*R*)-2-hydroxy-3-methylbutanoyl-*N*-methyl-*L*-leucyl] $_4$ , is produced by *Beauveria bassiana* and *Verticillium lecanii*,<sup>12,16</sup> whereas PF1022 group compounds (types A–H) are produced by *Rosellinia* sp.<sup>13,17–20</sup> The structure of PF1022A is cyclo[(2*R*)-2-hydroxypropanoyl-*N*-methyl-*L*-leucyl-3-phenyl-(2*R*)-2-hydroxypropanoyl-*N*-methyl-*L*-leucyl] $_2$ . Therefore, fungal compounds have *R*-form hydroxy acids and *L*-form amino acids, which is in accordance with the stereochemistry of **1**. Bassianolide has an insecticidal activity, and is reported to inhibit the tonic component of contraction induced by acetylcholine in the guinea pig.<sup>21</sup> Emodepside, a semisynthetic derivative of PF1022A, with a morpholine ring at each of the two phenyl rings in the para position, is now used as a nematocide in the veterinary field. The target of emodepside and PF1022A is suggested to be depsiphilin, which is similar to mammalian latrophilin, a presynaptic G-protein-coupled receptor.<sup>22</sup>

### Biological activities

Compound **1** showed ryanodine-binding inhibition against cockroach RyR with an  $\text{IC}_{50}$  value of 4.2  $\mu\text{M}$  (Table 3). The  $\text{IC}_{50}$  value of ryanodine-binding inhibition against mouse leg muscle RyR was 53.9  $\mu\text{M}$ , indicating that it was more than 10 times weaker when compared with the cockroach receptor. Ryanodine did not show high selectivity between cockroach and mouse receptors.

Compound **1** belongs to a group of cyclic depsipeptides having alternately combined amino acids and hydroxy acids. However, **1** is the first compound that has small side-chain amino acids and very

**Table 3** Ryanodine-binding inhibition of verticilide (**1**) and the other cyclic depsipeptides

Compound	Cockroach RyR	Mouse RyR
	$\text{IC}_{50}$ ( $\mu\text{M}$ )	$\text{IC}_{50}$ ( $\mu\text{M}$ )
Verticilide ( <b>1</b> )	4.2	53.9
Bassianolide	> 100	–
PF1022A	> 100	–
Beauvericin	> 100	–
Enniatin A	> 100	–
Enniatin A1	> 100	–
Enniatin B	> 100	–
Enniatin B1	> 100	–
Enniatin D	> 100	–
Enniatin F	> 100	–
Ryanodine <sup>a</sup>	0.03	0.07

Abbreviations: RyR, ryanodine receptor; –, not tested.

<sup>a</sup>Inhibitory activity of ryanodine is shown for comparison.

long, straight side-chain hydroxy acids, and hence its biological activity is interesting. We consequently studied the ryanodine-binding inhibitory activity of some cyclic depsipeptides—bassianolide, PF1022A, beauvericin, and enniatins A, A1, B, B1, D and F (Figure 1). Beauvericin and the enniatins are 18-membered ring depsipeptides. All depsipeptides showed no ryanodine-binding inhibition against cockroach RyR at 100  $\mu\text{g ml}^{-1}$ . Therefore, it is likely that the unique side-chain structure in **1** could contribute to its ryanodine-binding inhibition.

Insecticidal and nematocidal activities of **1** were studied by a microplate assay using brine shrimp, *Artemia salina*, and the free-living nematode *Caenorhabditis elegans*. The minimum growth inhibitory concentrations of **1** against *C. elegans* and *A. salina* were both 20  $\mu\text{g ml}^{-1}$ . Compound **1** did not inhibit the growth of P388 cells at 100  $\mu\text{g ml}^{-1}$  and it did not show any antimicrobial activity against some Gram-positive and Gram-negative bacteria, fungi and yeasts at 10  $\mu\text{g disk}^{-1}$  (paper disk method).

## EXPERIMENTAL SECTION

### General

NMR spectra were recorded on a Varian Inova 600 spectrometer (Varian Inc., Palo Alto, CA, USA) ( $^2\text{-}^3\text{J}_{\text{CH}}=8$  Hz in heteronuclear multiple bond correlation). Chemical shifts are shown in  $\delta$  values (p.p.m.) relative to chloroform at 7.26 p.p.m. for  $^1\text{H}$  NMR and at 77.0 p.p.m. for  $^{13}\text{C}$  NMR. MS was conducted on a JEOL JMS-AX505 HA spectrometer (JEOL Ltd., Akishima, Japan). The UV and IR spectra were measured with a Shimadzu UV-240 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) and a Horiba FT-210 Fourier (Horiba Ltd., Kyoto, Japan) transform infrared spectrometer, respectively. Optical rotations were recorded on a JASCO model DIP-181 polarimeter (JASCO Corporation, Hachioji, Japan).

### Taxonomy of the producing organism

Morphological observations of the verticilide-producing strain were carried out using an Olympus Vanox-S AH-2 microscope (Olympus Corporation, Tokyo, Japan).

### Media

The seed medium consisted of glucose 2.0%, Polypepton (Nihon Pharmaceutical Co. Ltd, Tokyo, Japan) 0.5%, yeast extract (Oriental Yeast Co. Ltd., Tokyo, Japan) 0.2%,  $\text{KH}_2\text{PO}_4$  0.1%,  $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$  0.05% and agar 0.1%, pH 6.0. The production was carried out in 1-l Roux flasks, each containing Italian rice 150 g,  $\text{FeSO}_4\cdot 7\text{H}_2\text{O}$  0.9 mg,  $\text{MnCl}_2\cdot 4\text{H}_2\text{O}$  0.9 mg,  $\text{ZnSO}_4\cdot 7\text{H}_2\text{O}$  0.9 mg,  $\text{CuSO}_4\cdot 5\text{H}_2\text{O}$  0.9 mg,  $\text{CoCl}_2\cdot 6\text{H}_2\text{O}$  0.9 mg and tap water (90 ml).

### Acid hydrolysis of 1

Compound 1 (50.0 mg) was hydrolyzed with 6 M hydrochloric acid (HCl; 2.5 ml) at 110 °C for 24 h in a sealed tube. The reaction mixture was concentrated under reduced pressure. The residue was applied to a Dowex 50W $\times$ 8 column ( $\text{H}^+$  form,  $\phi$  1.7 $\times$ 9.8 cm) and eluted with water and 1 M ammonia. The water eluate was concentrated to dryness to yield a white powder of 3 (20.2 mg). The ammonia eluate was concentrated to yield a colorless oil of 2 (12.4 mg).

*N*-Methyl-L-alanine (2).  $[\alpha]_{\text{D}}^{27} +6.44$  ( $c$  0.56, 6 M HCl); FAB-MS ( $m/z$ ) 104 ( $\text{M}+\text{H}^+$ );  $^1\text{H}$  NMR (600 MHz,  $\text{D}_2\text{O}$ )  $\delta$  3.45 (1H, q,  $J_{\alpha,\beta}=6.6$  Hz,  $\alpha\text{-CH}$ ), 2.53 (3H, s,  $\text{NCH}_3$ ), 1.31 (3H, d,  $J_{\alpha,\beta}=6.6$  Hz,  $\beta\text{-CH}_3$ );  $^{13}\text{C}$  NMR (150 MHz,  $\text{D}_2\text{O}$ )  $\delta$  175.1 ( $\text{C}=\text{O}$ ), 59.1 ( $\alpha\text{-CH}$ ), 31.2 ( $\text{NCH}_3$ ), 14.7 ( $\beta\text{-CH}_3$ ). The stereochemistry was found to be the *l*-form by chiral HPTLC (CHIR, Merck 114101; developed by 0.05 M  $\text{KH}_2\text{PO}_4$ -methanol- $\text{CH}_3\text{CN}$  (5:5:1)).

(2*R*)-2-Hydroxyheptanoic acid (3).  $[\alpha]_{\text{D}}^{25} -15.46$  ( $c$  1.0,  $\text{CHCl}_3$ ); HR-FAB-MS ( $m/z$ ) found 145.0866, calcd 145.0865 [ $\text{M}-\text{H}$ ] $^-$ ,  $\text{C}_7\text{H}_{13}\text{O}_3$ ];  $^1\text{H}$  NMR (600 MHz,  $\text{D}_2\text{O}$ )  $\delta$  4.12 (1H, m,  $\alpha\text{-CH}$ ), 1.65 (1H, m,  $\beta\text{-CH}_2$ ), 1.55 (1H, m,  $\beta\text{-CH}_2$ ), 1.24 (2H, m,  $\gamma\text{-CH}_2$ ), 1.15 (4H, m,  $\delta\text{-CH}_2$  and  $\epsilon\text{-CH}_2$ ), 0.71 (3H, m,  $\zeta\text{-CH}_3$ );  $^{13}\text{C}$  NMR (150 MHz,  $\text{D}_2\text{O}$ )  $\delta$  178.6 ( $\text{C}=\text{O}$ ), 70.6 ( $\alpha\text{-CH}$ ), 33.5 ( $\beta\text{-CH}_2$ ), 30.9 ( $\delta\text{-CH}_2$ ), 24.0 ( $\gamma\text{-CH}_2$ ), 22.1 ( $\epsilon\text{-CH}_2$ ), 13.6 ( $\zeta\text{-CH}_3$ ).

### Alkaline hydrolysis of 1

After addition of 1 M sodium hydroxide (1.6 ml) to a solution of 1 (50.0 mg) in methanol (5.0 ml), the mixture was stirred for 24 h at room temperature. The reaction mixture was neutralized with 1 M HCl and concentrated under reduced pressure. The concentrate was purified using HPLC on a Senshu Pak  $\text{C}_{18}$  column ( $\phi$  20 $\times$ 250 cm, Senshu Scientific Co. Ltd., Tokyo, Japan) with  $\text{CH}_3\text{CN}$ -10% phosphoric acid (gradient from 15:85 to 30:70) at 40 °C. The eluate at 32 min was concentrated and partitioned between ethyl acetate and  $\text{H}_2\text{O}$ . The ethyl acetate layer was concentrated to dryness to yield a white powder of 4 (3.1 mg).

(2*S*)-2-Hydroxyheptanoyl-*N*-methyl-L-alanine (4). HR-FAB-MS ( $m/z$ ) found 232.1559, calcd 232.1549 [ $\text{M}+\text{H}$ ] $^+$ ,  $\text{C}_{11}\text{H}_{22}\text{NO}_4$ ];  $^1\text{H}$  NMR (600 MHz,  $\text{CD}_3\text{OD}$ )  $\delta$  4.99 (1H, m,  $\text{H}^{\text{H}\alpha}\alpha\text{-CH}$ ), 4.28 (1H, q,  $J_{\alpha,\beta}=6.9$  Hz,  $\text{Ala}\alpha\text{-CH}$ ), 2.97 (3H, s,  $\text{Ala}\text{NCH}_3$ ), 2.03 (1H, m,  $\text{H}^{\text{H}\beta}\beta\text{-CH}_2$ ), 1.88 (1H, m,  $\text{H}^{\text{H}\beta}\beta\text{-CH}_2$ ), 1.55 (3H, d,  $J_{\alpha,\beta}=6.9$  Hz,  $\text{Ala}\beta\text{-CH}_3$ ), 1.47 (2H, m,  $\text{H}^{\text{H}\gamma}\gamma\text{-CH}_2$ ), 1.36 (4H, m,  $\text{H}^{\text{H}\delta}\delta\text{-CH}_2$  &  $\text{H}^{\text{H}\epsilon}\epsilon\text{-CH}_2$ ), 0.92 (3H, t,  $J_{\epsilon,\zeta}=7.2$  Hz,  $\text{H}^{\text{H}\zeta}\zeta\text{-CH}_3$ );  $^{13}\text{C}$  NMR (150 MHz,  $\text{CD}_3\text{OD}$ )  $\delta$  170.1 ( $\text{Ala}\text{C}=\text{O}$ ), 167.6 ( $\text{H}^{\text{H}\alpha}\alpha\text{-C}=\text{O}$ ), 78.5 ( $\text{H}^{\text{H}\alpha}\alpha\text{-CH}$ ), 57.9 ( $\text{Ala}\alpha\text{-CH}$ ), 32.6

( $\text{H}^{\text{H}\delta}\delta\text{-CH}_2$ ), 32.1 ( $\text{H}^{\text{H}\beta}\beta\text{-CH}_2$ ), 32.0 ( $\text{Ala}\text{NCH}_3$ ), 25.1 ( $\text{H}^{\text{H}\gamma}\gamma\text{-CH}_2$ ), 23.5 ( $\text{H}^{\text{H}\epsilon}\epsilon\text{-CH}_2$ ), 16.5 ( $\text{Ala}\beta\text{-CH}_3$ ), 14.3 ( $\text{H}^{\text{H}\zeta}\zeta\text{-CH}_3$ ).

### Ryanodine-binding assay

Cockroach membrane containing RyR was prepared as reported previously.<sup>6</sup> The membrane of mouse leg muscle containing RyR was prepared as follows: mouse leg muscle (2.15 g) was cut and washed with 11 ml of preparation buffer (50 mM Tris-HCl, pH 8.0 containing 4  $\mu\text{g ml}^{-1}$  each of aprotinin, leupeptin and pepstatin). It was homogenized and washed with 7 ml of the buffer. The homogenate was centrifuged (2000 g) for 10 min at 4 °C, washed and the supernatant was centrifuged again (25 000 g) for 45 min. The pellets were re-suspended in 1.7 ml of the buffer and stored at -80 °C until the binding assay.

For the binding assay, sample solutions were put into individual wells of a microplate. The samples were mixed with 10  $\mu\text{l}$  ethanol solution containing 0.1 nM ryanodine, and 70  $\mu\text{l}$  of the buffer, which consisted of 50 mM Tris-HCl (pH 8.0), 300  $\mu\text{M}$  sucrose, 500  $\mu\text{M}$   $\text{CaCl}_2$ , 1.5 M KCl, 4  $\mu\text{g ml}^{-1}$  each of aprotinin, leupeptin and pepstatin, with 10  $\mu\text{l}$  of 20 nM [ $^3\text{H}$ ]ryanodine (56.9 Ci mmol $^{-1}$ , PerkinElmer NET-950, PerkinElmer Inc, Waltham, MA, USA) solution, was also added to the microplate. The reaction was started by adding each of 10  $\mu\text{l}$  of the RyR-containing membrane (2.5 mg protein per ml) described above, with the microplate subsequently being shaken for 90 min at room temperature. Free ligand was separated from the protein-bound ligand by filtration through a glass-fiber filter (PerkinElmer Printed Filtermat B) using Inotech cell harvester (Inotech Biosystems International Inc., Rockville, MD, USA), followed by washing with ice-cold 20 mM Tris-HCl buffer (pH 8.0, 3 $\times$ 300  $\mu\text{l}$ ). The filter was dried in a microwave oven, a solid scintillator sheet (PerkinElmer MeltiLex B/HS) was put on the filter and the sheet was melted on a hot plate. The activity retained on the filter was measured using PerkinElmer MicroBeta TriLux liquid scintillation counter. Specific binding was determined as the difference between total binding ([ $^3\text{H}$ ]ryanodine) and non-specific binding ([ $^3\text{H}$ ]ryanodine plus 500-fold excess of unlabelled ryanodine).

### Other biological assays

The assay method for nematocidal and insecticidal activities was carried out using previously reported procedures.<sup>23</sup>

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