Tetrahedron 69 (2013) 3536–3542

Contents lists available at SciVerse ScienceDirect

Tetrahedron

journal homepage: www.elsevier.com/locate/tet

Terresterol, a polyoxygenated lanostanoid, isolated from the oomycete *Saprolegnia terrestris*, and its innate immune-promoting activity

Haruhisa Kikuchi *, Yuichi Sato, Shoichiro Kurata, Yasuhiro Katou, Yoshiteru Oshima

Graduate School of Pharmaceutical Sciences, Tohoku University, Aoba-yama, Aoba-ku, Sendai 980-8578, Japan

ARTICLE INFO

Article history:
Received 31 January 2013
Received in revised form 19 February 2013
Accepted 23 February 2013
Available online 1 March 2013

Keywords:
Oomycetes
Innate immunity
Immunostimulating agents
Lanostanoid
Structural elucidation

ABSTRACT

Oomycetes are widely spread eukaryotic microorganisms. However, there have only been a few reports on the secondary metabolites produced by them. In the course of screening for innate immune regulators from natural resources, we isolated a new polyoxygenated lanostanoid, terresterol (**1**), from the oomycetes *Saprolegnia terrestris*. Compounds bearing a 21,22,24,25-tetraoxygenated side chain similar to **1** have only been rarely described, and the isolation of such compounds shows that oomycetes are promising resources for natural product chemistry. In addition, terresterol (**1**) has potential to be used as a lead compound for novel immunostimulating agents against bacterial infections and as probe for the innate immune system.

1. Introduction

Oomycetes are eukaryotic microorganisms that are widely found in water and soil.[1] Since they were observed to form fungus-like mycelium, they were classified as fungi in the past. However, the cell walls of them are composed of cellulose rather than chitin, as in the fungi.[2] In the present, ultrastructures, biochemistry, and genomic sequences indicate that oomycetes belong to the Kingdom Chromista, not the Kingdom Fungi, and are related to organisms such as brown algae and diatoms. Because many species of oomycetes have plant pathogenicity and cause plant diseases, they are agriculturally and economically important.[3] However, they have not been exploited in natural product chemistry. Furthermore, there have only been reports on a few compounds produced by these organisms, including antheridiol, oogoniol and their derivatives, a sex hormone in oomycetes *Achlya*.[4]

Recently, we have focused on innate immunity, and we established an ex vivo culture system based on the *Drosophila* IMD signaling pathway to screen pharmaceuticals that target innate immunity.[5] Because of the striking conservation between the mechanisms that regulate insect immunity and mammalian innate immunity,[6,7] *Drosophila* is a model organism for genetic and molecular studies of innate immunity, and our culture system has proven to be useful for identifying immune regulators that act on human innate immunity.[5] We have used this system to search for natural substances that regulate innate immunity.[8] Here we describe the isolation of terresterol (**1**) (Fig. 1), a polyoxygenated lanostanoid from the oomycetes, *Saprolegnis terrestris*, which also functions as an innate immune promoter.

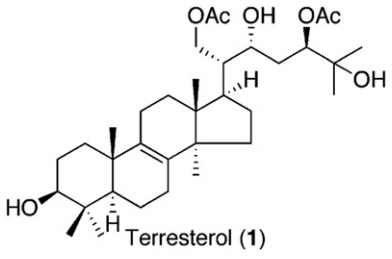


Fig. 1. Structure of terresterol (**1**).

2. Results and discussion

2.1. Isolation

S. terrestris NRBC102124 was cultivated in yeast extract–malt extract broth. The mycelial cake of culture broth (9.0 L) was

* Corresponding author. Tel.: +81 22 795 6824; fax: +81 22 795 6821; e-mail address: hal@mail.pharm.tohoku.ac.jp (H. Kikuchi).

0040-4020/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.tet.2013.02.088

lyophilized and extracted with methanol at room temperature to yield an extract (9.86 g). The ethyl acetate solubles (1.47 g) in the methanol extract were separated by repeated column chromatography over silica gel and ODS column to yield terresterol (**1**) (25 mg) along with fucosterol and 24-methylenecholesterol.

2.2. Structural elucidation

HRFABMS (*m*/*z* 577.4078 $[M+H]^+$ and *m*/*z* 599.3901 $[M+Na]^+$) indicated the molecular formula of **1** as $C_{34}H_{56}O_7$. The ^{13}C NMR spectrum of **1** showed the presence of two ester carbonyl, two quaternary olefinic, one oxygenated quaternary, three oxymethine, one oxymethylene, four quaternary, three methine, nine methylene, and nine methyl carbons (Table 1). The cross-peaks in $^1H-^1H$ COSY implied that C-1–C-2–C-3, C-5–C-6–C-7, C-11–C-12, C-15–C-16–C-17–C-20–C-21, and C-22–C-23–C-24 were connected (Fig. 2A). HMBC correlations for H-22 to C-21; H_2-21 to C-22; H_3-26 to C-24 and C-25; and H_3-27 to C-24 indicated a side chain moiety consisting of C-20–C-27. HMBC correlations were observed for H_2-21 and H_3-32 to C-31; H-24 and H_3-34 to C-33, suggesting that an acetoxy group was attached to C-21 and C-24, respectively. The existence of a cyclopenta[*a*]phenanthrene skeleton, except at the bond between C-8 and C-9, was inferred from the HMBC correlations for H_2-6 to C-8; H_2-12 to C-9; H_3-18 to C-12, C-13, C-14 and C-17; H_3-19 to C-1, C-5, C-9 and C-10; H_3-28 to C-3, C-4 and C-5; H_3-29 to C-3, C-4 and C-5; H_3-30 to C-8, C-13, C-14 and C-15. Finally, two remaining olefinic carbons, C-8 and C-9, were supposed to be connected, and the planar structure of **1** was elucidated as 21,24-diacetoxylanostan-3,22,25-triol.

Table 1
^1H and ^{13}C NMR spectra of terresterol (**1**)

	Terresterol (**1**)[a]	
	^{13}C	^1H
1	35.5	1.61–1.67 (1H, m) 1.11–1.16 (1H, m)
2	27.6	1.56–1.63 (1H, m) 1.47–1.52 (1H, m)
3	78.8	3.15 (1H, dd, *J*=11.7, 4.5 Hz)
4	38.8	
5	50.3	0.97 (1H, dd, *J*=12.7, 1.8 Hz)
6	18.1	1.57–1.64 (1H, m) 1.41–1.46 (1H, m)
7	26.4	1.90–2.02 (2H, m)
8	133.9	
9	134.6	
10	37.0	
11	20.9	1.82–1.91 (2H, m)
12	29.9	1.55–1.64 (1H, m) 1.33–1.42 (1H, m)
13	44.5	
14	49.4	
15	30.7	1.53–1.59 (1H, m) 1.10–1.14 (1H, m)
16	26.7	1.72–1.77 (1H, m) 1.32–1.38 (1H, m)
17	42.5	1.73–1.79 (1H, m)
18	15.9	0.67 (3H, s)
19	19.1	0.90 (3H, s)
20	45.6	1.86–1.91 (1H, m)
21	63.0	4.26 (1H, dd, *J*=11.7, 2.9 Hz) 4.07 (1H, dd, *J*=11.7, 5.4 Hz)
22	68.4	3.55 (1H, br d, *J*=12.2 Hz)
23	32.1	1.55–1.69 (2H, m)
24	77.9	4.86 (1H, dd, *J*=9.5, 3.1 Hz)
25	71.7	
26	25.8	1.14 (3H, s)
27	25.7	1.14 (3H, s)
28	27.9	0.93 (3H, s)
29	15.3	0.74 (3H, s)
30	24.4	0.83 (3H, s)
31	171.1	
32	21.0	1.99 (3H, s)
33	171.9	
34	21.0	2.06 (3H, s)

[a] 600 MHz for ^1H and 150 MHz for ^{13}C in $CDCl_3$.

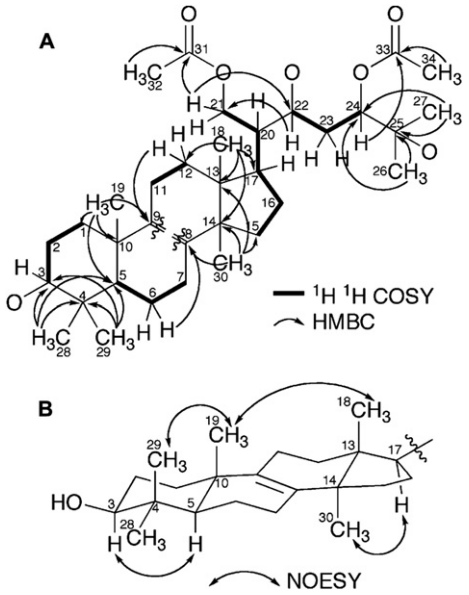


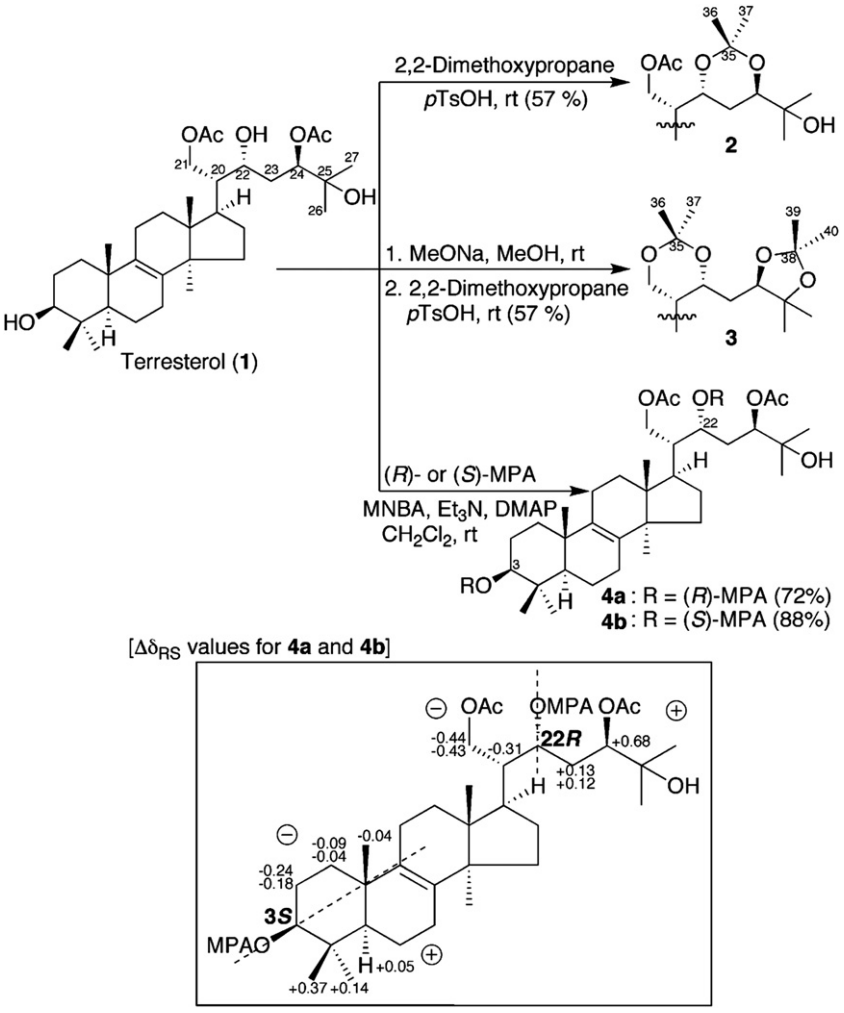
Fig. 2. Structural elucidation of terresterol (**1**). A. Planar structure of **1**. B. Relative structure of the tetracyclic moiety of **1**.

The cross-peaks of H_3-18–H_3-19[13] and H_3-19–H_3-29 in the NOESY spectrum of **1** indicated that the configurations of three methyl groups at C-18, C-19, and C-29 were β-axial (Fig. 2B). The cross-peaks of H-3–H-5 and H-17–H_3-30 revealed that the orientations of protons at C-3, C-5, C-17, and the 30-methyl group were α-axial. Thus, the relative configuration of the tetracyclic moiety of **1** was determined as 3*S**, 5*R**, 10*S**, 13*R**, 14*R**, and 17*R**. The relative configuration of the side chain moiety consisting of C-21–C-27 could not be directly established because of the flexibility of bond rotation.

To determine its relative configuration of the side chain, we have fixed its conformation. When terresterol (**1**) was treated with 2,2-dimethoxypropane under acidic conditions, the acetoxy group at C-24 was hydrolyzed and a 22,24-*O*-isopropylidene derivative **2** was afforded (Scheme 1). The NOESY spectrum of **2** showed the cross-peaks of H-22–H_3-36 and H-24–H_3-37 (Fig. 3A). In the ^1H NMR spectrum of **2**, the signal of H-24 (δ 3.99, d, *J*=4.4 Hz) implied that the dihedral angle between H-24 and H-23β was almost 90° (Fig. 3B). These facts indicated that the conformation of the 1,3-dioxane moiety was the twisted-boat form due to an alleviation of 1,3-diaxial interactions,[9] and the relative configurations of C-22 and C-24 were *R** and *R**, respectively.

After deacetylation of **1** using sodium methoxide, the treatment with 2,2-dimethoxypropane under acidic conditions afforded a 21,22:24,25-di-*O*-isopropylidene derivative **3** (Scheme 1). The NOESY spectrum of **3** showed the cross-peak between H-21β and H-22 (Fig. 4). In the ^1H NMR spectrum, the signals of H-21α and H-21β were observed at δ 4.00 (dd, *J*=11.6, 3.9 Hz) and 3.93 (dd, *J*=11.6, 3.7 Hz), respectively. These facts indicate that H-20 was an equatorial proton in the chair-formed 1,3-dioxane moiety, and the relative configuration of C-20 was *R**.

Since the relative structures of the tetracyclic moiety and the side chain moiety were determined separately, the absolute structures of these moieties were also separately determined. We employed the esterification of **1** with (*R*)-α-methoxyphenylacetic acid in the presence of MNBA[10] to yield 3*O*,22*O*-(*R*)-MPA diester **4a** (Scheme 1). In a similar manner, (*S*)-MPA diester **4b** was produced.



Scheme 1. Conversion of terresterol (**1**) into **2**, **3**, **4a**, and **4b**.

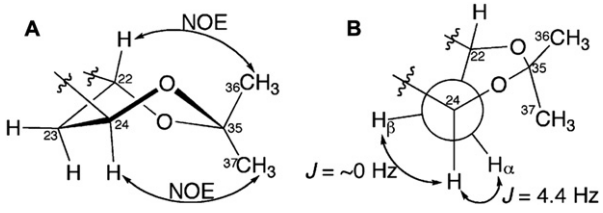


Fig. 3. Relative structure of the 22,24-*O*-isopropylidene derivative **2**. A. Conformation of the 1,3-dioxane moiety. B. Newman projection for the C-24–C-23 bond.

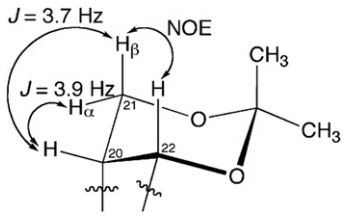


Fig. 4. Conformation of the 1,3-dioxane moiety of the 21,22:24,25-di-*O*-isopropylidene derivative **3**.

The $\Delta\delta_{RS}$ value of each proton was calculated from the difference in the chemical shifts of **4a** and **4b** in ^{1}H NMR spectra, and the structure of **1** was fit into the proposed model of α-methoxyphenylacetate[11] in accordance with the sign of $\Delta\delta_{RS}$. As a result, C-3 and C-22 had *S*- and *R*-configurations, respectively, and the absolute configuration of **1** was determined to be 3*S*, 5*R*, 10*S*, 13*R*, 14*R*, 17*R*, 20*R*, 22*R*, 24*R*.

2.3. Promoting activity on innate immune response

We evaluated the effect of terresterol (**1**) on the innate immune response using the ex vivo *Drosophila* culture system as previously described.[5] Although compound **1** showed cytotoxicity against *Drosophila* S2 cells (CC_{50} 40 μg/mL), 10 μg/mL of **1** increased the *Drosophila* innate immune response (Fig. 5A) more than two-fold. Furthermore, in order to evaluate the innate immune activity of **1** at the cellular level, *Drosophila* S2 cells, which were stably transfected with *luciferase* reporter gene driven by *attacin* promoter, were used.[12] Attacin is one of the antimicrobial peptides regulated by the *Drosophila* IMD pathway. Using *attacin* reporter, compound **1** showed concentration-dependent promoting activity at up to 0.1 μg/mL dosage (Fig. 5B).

2.4. Derivatization of terresterol (1)

To reveal the structural requirements of terresterol (**1**) for the innate immune-promoting activity, we synthesized several derivatives from **1**. By the treatment of **1** with sodium methoxide, both of the 22- and 24-acetyl groups were removed to produce a pentaol **5** (Scheme 2). A 3,21,22,24-tetraacetate **6** was afforded by

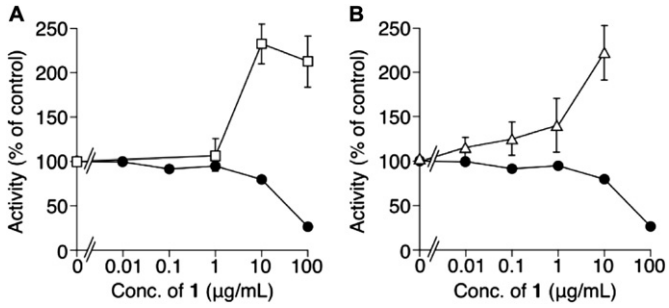
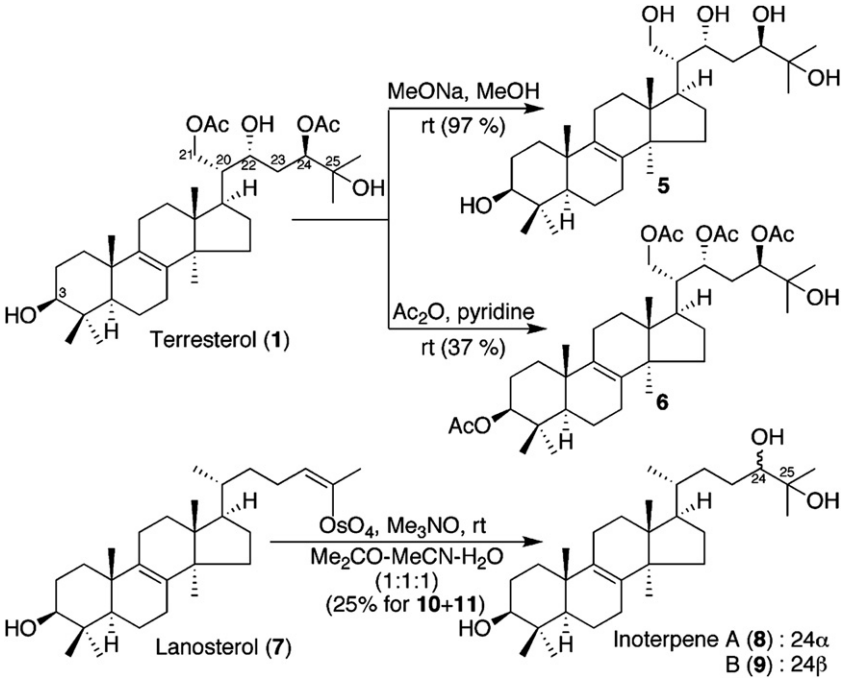


Fig. 5. Innate immune-promoting activity of terresterol (**1**). A. Effects of terresterol (**1**) on DAP-type peptidoglycans-mediated activation of *Drosophila Dpt-lacZ*. DAP-type peptidoglycans-mediated activation of *Dpt-lacZ* (open squares) and *Drosophila* S2 cell viability (black circles) are represented as the percent relative to the control (DMSO). B. Effects of terresterol (**1**) on *attacin* promoter activity in *Drosophila* S2 cells. S2 cells stably transfected with a *luciferase* reporter gene driven by an *attacin* promoter were treated with terresterol (**1**) at indicated concentrations, and stimulated by DAP-type peptidoglycan. The luciferase activity (open triangles) and *Drosophila* S2 cell viability (black circles) are represented as the percent relative to the control (DMSO). The bars indicate the standard errors of three independent measurements.

the acetylation of **1**. Dihydroxylation of lanosterol (**7**), using osmium tetroxide, yielded a 1:1 mixture of inoterpene A (**8**) and B (**9**), which were reported as the constituents of the fungus *Inonotus obliquus*.[13] The activities of these compounds on the *Drosophila* innate immune response were evaluated by the use of *attacin* reporter (Fig. 6). For compound **6**, the innate immune response was completely inhibited at 10 μg/mL. However, the viability of *Drosophila* S2 cells was reduced to 15% of control at the same concentration, indicating that compound **6** showed only cytotoxicity, and not inhibitory activity on innate immune response. The activity of the mixture of inoterpene A (**8**) and B (**9**) was almost the same as that of **6**. On the other hand, the deacetylated derivative **5** inhibited the activity on *Att-Luc* system in a concentration-dependent manner (IC_{50} 1.7 μg/mL). Since the CC_{50} value (23 μg/mL) for S2 cells was 10 times higher than the IC_{50} value for the *Att-Luc* system, compound **5** had selective immunosuppressive effects. These results implied that the hydroxy groups and acetoxy groups in the side chain of **1** were necessary for regulating innate immune responses, and the number of acetoxy groups may switch over from a promoting activity to an inhibitory activity.



Scheme 2. Synthesis of **5**, **6**, **8**, and **9**.

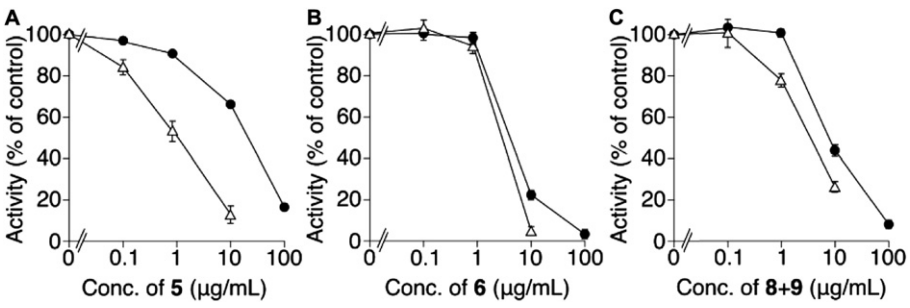


Fig. 6. Effects of compounds **5** (A), **6** (B), and the mixture of **8** and **9** (C) on *attacin* promoter activity in *Drosophila* S2 cells. The luciferase activity (open triangles) and *Drosophila* S2 cell viability (black circles) are represented as the percent relative to the control (DMSO). The bars indicate the standard errors of three independent measurements.

Although many polyoxygenated lanostanoids and tetracyclic triterpenoids have been reported, compounds bearing a 21,22,24,25-tetraoxygenated side chain similar to terresterol (**1**) have been rarely described, as is the case with 1α,7α-diacetoxyapotirucall-14-ene-3α,21,22,24,25-pentaol (**10**) (Fig. 7), which was isolated from *Azadirachta indica*.[14] Therefore, the isolation of such compounds shows that oomycetes are promising resources for natural product chemistry. Additionally, the isolation of fucosterol and 24-methylenecholesterol supports the phylogenetic relationship between oomycetes and brown algae.[15]

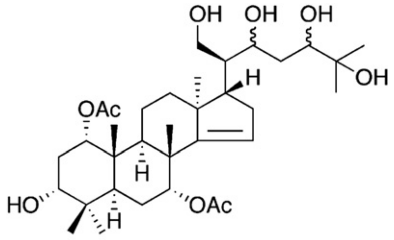


Fig. 7. Structure of 1α,7α-diacetoxyapotirucall-14-ene-3α,21,22,24,25-pentaol (**10**).

Terresterol (**1**) showed innate immune-promoting activity. Thus, terresterol (**1**) has potential to be used as a lead compound for novel immunostimulating agents against bacterial infections and as probes for the innate immune system.

3. Experimental section

3.1. General methods

Analytical TLC was performed on silica gel 60 F_{254} (Merck). Column chromatography was carried out on silica gel 60 (70–230 mesh, Merck). NMR spectra were recorded on JEOL ECA-600 and AL-400. Chemical shifts for ^{1}H and ^{13}C NMR are given in parts per million (δ) relative to tetramethylsilane (δ_H 0.00) and residual solvent signals (δ_C 77.0 and 49.0 for $CDCl_3$ and CD_3OD, respectively) as internal standards. Mass spectra were measured on JEOL JMS-700 and JMS-DX303. IR spectra were measured on JASCO FT/IR-4200.

3.2. Organism and culture conditions

The oomycete, *S. terrestris* NRBC102124 was supplied by Biological Resource Center (NBRC), National Institute of Technology and Evaluation, Chiba, Japan. This strain was cultured in YM medium (glucose 1%, Bacto Peptone 0.5%, Bacto Malt extract 0.3%, yeast extract 0.3%) at 28 °C for 2 weeks on a rotary shaker at 150 rpm.

3.3. Isolation of terresterol (1)

The mycelia of *S. terrestris*, which was obtained from the cultured broth (9.0 L), were extracted three times with methanol (2 L) at room temperature to give the extract (9.86 g). This extract was partitioned with ethyl acetate and water to yield ethyl acetate solubles (1.47 g). The ethyl acetate solubles were chromatographed over SiO_2, and the column eluted with *n*-hexane/ethyl acetate mixtures with increasing polarity to afford hexane/ethyl acetate (2:1) eluent (fraction A, 750 mg) and hexane/ethyl acetate (1:9) eluent (fraction B, 54 mg). Fraction A was further separated by ODS column using water/acetonitrile solvent system to give the mixture of fucosterol and 24-methylenecholesterol (1:1) (25.5 mg). Fraction B was also further separated by ODS column using water–acetonitrile solvent system to give terresterol (**1**) (25.3 mg) as water/acetonitrile (3:7) elutant.

Data for **1**: colorless amorphous solid; mp 252–255 °C (dec); $[\alpha]_D$ +27.1 (*c* 0.461, $CHCl_3$); IR (film) ν_{max} (cm^{-1}) 3416, 2935, 1737, 1243; ^{1}H NMR and ^{13}C NMR data are shown in Table 1; HRFABMS *m*/*z* 599.3901 $[M+Na]^+$ (599.3924 calcd for $C_{34}H_{56}O_7Na$) and 577.4078 $[M+H]^+$ (577.4101 calcd for $C_{34}H_{57}O_7$).

3.4. Conversion of 1 into 22,24-*O*-isopropylidene derivative 2

To a solution of **1** (1.2 mg, 2.1 μmol) in 2,2-dimethoxypropane (2 mL) was added *p*-toluenesulfonic acid (2.1 mg). After being stirred for 14 h at room temperature, the reaction mixture was poured into saturated sodium bicarbonate solution, and extracted with ethyl acetate three times. The combined organic layer was washed with water and brine, dried over sodium sulfate, and evaporated. The residue was chromatographed over silica gel eluted by hexane/ethyl acetate (1:1) to give **2** (0.6 mg, 1.1 μmol, 57%). Data for **2**: colorless amorphous solid; ^{1}H NMR (600 MHz, $CDCl_3$) δ 4.29 (1H, dt, *J*=11.8, 3.8 Hz), 4.21 (2H, d, *J*=2.1 Hz), 3.99 (1H, d, *J*=3.2 Hz), 3.20 (1H, dd, *J*=11.8, 4.3 Hz), 1.88–2.09 (8H, m), 1.45–1.76 (14H, m), 1.38 (3H, s), 1.27 (3H, s), 1.18–1.36 (7H, m), 1.02 (1H, dd, *J*=12.7, 2.1 Hz), 0.98 (3H, s), 0.95 (3H, s), 0.88 (3H, s), 0.79 (3H, s), 0.72 (3H, s); ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.0, 134.8, 134.0, 118.9, 80.7, 79.3, 79.0, 69.1, 63.0, 50.4, 49.5, 44.6, 43.1, 41.6, 38.9, 37.1, 35.6, 30.9, 29.7, 28.0 (2C), 27.9, 27.4, 27.0, 26.5, 25.7, 24.5, 23.5, 21.2, 21.1, 20.4, 19.2, 18.2, 16.0, 15.4; HRFABMS *m*/*z* 575.4332 $[M+H]^+$ (575.4312 calcd for $C_{35}H_{59}O_6$).

3.5. Conversion of 1 into 21,22:24,25-di-*O*-isopropylidene derivative 3

To a solution of **1** (5.0 mg, 8.7 μmol) in methanol (1 mL) was added sodium methoxide (5.0 mg). After being stirred for 3 h at room temperature, the mixture was acidified with Dowex 50w (H^+ form) until the pH reached 2, and filtered. Then, the filtrate was evaporated, and the residue was dissolved in 2,2-dimethoxypropane (2 mL). *p*-Toluenesulfonic acid (4.0 mg) was added to the solution. After being stirred for 3 h at room temperature, the reaction mixture was poured into saturated sodium bicarbonate solution, and extracted with ethyl acetate three times. The combined organic layer was washed with water and brine, dried over sodium sulfate, and evaporated. The residue was chromatographed over silica gel eluted by hexane/ethyl acetate (9:1) to give **3** (3.1 mg, 5.4 μmol, 62% (two steps)). Data for **3**: colorless amorphous solid; ^{1}H NMR (600 MHz, $CDCl_3$) δ 4.19 (1H, d, *J*=11.0 Hz), 4.00 (1H, dd, *J*=11.6, 3.9 Hz), 3.93 (1H, dd, *J*=11.6, 3.7 Hz), 3.87 (1H, d, *J*=10.1 Hz), 3.15 (1H, dd, *J*=11.7, 4.4 Hz), 2.25 (1H, q, *J*=8.6 Hz), 1.94–2.08 (5H, m), 1.86 (1H, dt, *J*=13.4, 8.7 Hz), 1.72 (1H, dt, *J*=12.8, 2.9 Hz), 1.19–1.67 (12H, m), 1.40 (6H, s), 1.37 (3H, s), 1.31 (3H, s), 1.24 (3H, s), 1.07 (3H, s), 1.04 (1H, d, *J*=12.6 Hz), 0.99 (3H, s), 0.97 (3H, s), 0.93 (3H, s), 0.80 (3H, s), 0.72 (3H, s); ^{13}C NMR (150 MHz, $CDCl_3$) δ 134.9, 134.0, 106.4, 99.0, 79.9, 79.6, 79.0, 71.4, 63.9, 50.4, 49.4, 45.3, 42.9, 40.6, 38.9, 37.0, 35.6, 34.0, 31.6, 30.6, 29.7, 29.1, 28.6, 28.0, 27.9, 26.8, 26.6, 25.7, 24.3, 23.1, 21.2, 21.0, 19.2, 18.2, 15.9, 15.4; HRFABMS *m*/*z* 572.4431 $[M]^+$ (572.4438 calcd for $C_{36}H_{60}O_5$).

3.6. Conversion of 1 into di-(*R*)-α-methoxyphenylacetate 4a

To a solution of **1** (1.1 mg, 1.9 μmol) in dichloromethane (0.5 mL) were added triethylamine (20 μL), DMAP (2.9 mg), 2-methyl-6-nitrobenzoic anhydride (8.2 mg), and (*R*)-α-methoxyphenylacetic acid (7.1 mg) at room temperature. After being stirred for 6 h, the mixture was poured into saturated ammonium chloride solution, and extracted with ethyl acetate three times. The combined organic layer was washed with water and brine, dried over sodium sulfate,

and evaporated. The residue was chromatographed over silica gel eluted by hexane/ethyl acetate (1:1) to give **4a** (1.2 mg, 1.4 μmol, 72%). Data for **4a**: colorless oil; ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.41–7.43 (2H, m), 7.36–7.39 (2H, m), 7.29–7.35 (6H, m), 4.94–4.97 (1H, m), 4.74–4.76 (1H, m), 4.73 (1H, s), 4.72 (1H, s), 4.52 (1H, dd, J=11.3, 4.7 Hz), 3.68 (1H, dd, J=11.9, 4.2 Hz), 3.62 (1H, dd, J=11.9, 2.8 Hz), 3.44 (3H, s), 3.41 (3H, s), 2.09 (3H, s), 1.98–2.02 (2H, m), 1.91 (3H, s), 1.17–1.86 (18H, m), 1.15 (3H, s), 1.14 (3H, s), 1.08 (1H, dd, J=12.7, 2.1 Hz), 0.90 (3H, s), 0.83 (3H, s), 0.82 (3H, s), 0.80 (3H, s), 0.55 (3H, s); HRFABMS: m/z 872.5081 $[M]^+$ (872.5071 calcd for $C_{52}H_{72}O_{11}$).

3.7. Conversion of 1 into di-(*S*)-α-methoxyphenylacetate 4b

In the same manner as the synthesis of **4a**, compound **4b** (1.2 mg, 1.4 μmol, 88%) was synthesized from **1**. Data for **4b**: colorless oil; ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.43–7.45 (4H, m), 7.29–7.38 (6H, m), 4.89–4.91 (1H, m), 4.72 (1H, s), 4.69 (1H, s), 4.49 (1H, dd, J=11.6, 4.3 Hz), 4.11 (1H, dd, J=12.1, 2.3 Hz), 4.02–4.07 (2H, m), 3.40 (3H, s), 3.38 (3H, s), 2.03 (3H, s), 2.02 (3H, s), 1.94–1.99 (2H, m), 1.14–1.81 (18H, m), 1.03 (1H, dd, J=12.7, 2.0 Hz), 0.94 (3H, s), 0.92 (3H, s), 0.91 (3H, s), 0.84 (3H, s), 0.68 (3H, s), 0.65 (3H, s), 0.42 (3H, s); HRFABMS: m/z 872.5032 $[M]^+$ (872.5071 calcd for $C_{52}H_{72}O_{11}$).

3.8. Conversion of 1 into pentaol 5

To a solution of **1** (2.2 mg, 3.8 μmol) in methanol (1 mL) was added sodium methoxide (5.0 mg). After being stirred for 3 h at room temperature, the mixture was acidified with Dowex 50w (H^+ form) until the pH reached 2, and filtered. Then, the filtrate was evaporated, and the residue was chromatographed over silica gel eluted by ethyl acetate/methanol (9:1) to give **5** (1.8 mg, 3.7 μmol, 97%). Data for **5**: colorless amorphous solid; ^{1}H NMR (400 MHz, CD_3OD) δ 3.96–4.02 (2H, m), 3.57–3.65 (2H, m), 3.14–3.18 (1H, m), 1.98–2.19 (5H, m), 1.42–1.79 (14H, m), 1.20–1.26 (1H, m), 1.18 (3H, s), 1.17 (3H, s), 1.05 (1H, dd, J=12.3, 1.7 Hz), 1.02 (3H, s), 0.98 (3H, s), 0.90 (3H, s), 0.82 (3H, s), 0.80 (3H, s); HRFABMS: m/z 493.3901 $[M+H]^+$ (493.3890 calcd for $C_{30}H_{53}O_5$).

3.9. Conversion of 1 into tetraacetate 6

To a solution of **1** (1.2 mg, 2.1 μmol) in pyridine (1 mL) was added acetic anhydride (300 μL). After being stirred for 5 h at room temperature, the reaction mixture was evaporated. The residue was chromatographed over silica gel eluted by hexane/ethyl acetate (3:2) to give **6** (0.6 mg, 0.9 μmol, 37%). Data for **6**: colorless amorphous solid; ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.91 (1H, dt, J=10.9, 3.0 Hz), 4.87 (1H, dd, J=10.6, 3.0 Hz), 4.49 (1H, dd, J=11.8, 4.5 Hz), 4.21 (1H, dd, J=11.8, 2.6 Hz), 4.11 (1H, dd, J=11.8, 5.1 Hz), 2.09 (3H, s), 2.06 (3H, s), 2.05 (3H, s), 2.03 (3H, s), 1.23–1.95 (20H, m), 1.20 (6H, s), 1.14 (1H, dd, J=12.5, 2.1 Hz), 1.00 (3H, s), 0.91 (3H, s), 0.88 (6H, s), 0.72 (3H, s); HRFABMS: m/z 660.4223 $[M+H]^+$ (660.4233 calcd for $C_{38}H_{60}O_9$).

3.10. Synthesis of the mixture of inoterpene A (8) and B (9)

To a solution of lanosterol (**7**) (31.6 mg, 74.2 μmol) in acetone/acetonitrile/water (1:1:1) (4 mL) was added trimethylamine *N*-oxide (15.9 mg) and 4% osmium tetroxide solution in water (100 μL). After being stirred for 24 h at room temperature, the reaction mixture was poured into 10% sodium thiosulfate solution, and extracted with ethyl acetate three times. The combined organic layer was washed with saturated sodium bicarbonate solution and brine, dried over sodium sulfate, and evaporated. The residue was chromatographed over silica gel eluted by hexane/ethyl acetate (1:1) to give the 1:1 mixture of **8** and **9** (8.5 mg, 18.5 μmol, 25%). Data for the mixture of **8** and **9**: colorless amorphous solid; ^{1}H NMR and ^{13}C NMR data were identical to those of **8** and **9** reported in the literature;[13] HRFABMS m/z 460.3895 $[M]^+$ (460.3914 calcd for $C_{30}H_{52}O_3$).

3.11. Ex vivo *Drosophila* culture assay

The detailed procedure was described previously.[5b] Briefly, the abdominal cavity of third-instar larva was opened using fine pincettes. Individual whole larval tissues were cultured in Schneider's *Drosophila* medium (Gibco-BRL, Invitrogen, Carlsbad, CA) containing 20% fetal bovine serum (Valley Biomedical, Winchester, VA) and 1% antibiotics/antimycotics (Gibco-BRL) in each well of a 96-well plate at 25 °C. For each condition, six females were cultured to produce six replicates. The test compounds were dissolved in DMSO and added to the culture medium. To determine the effects of the test compounds on the innate immune response, *Dpt-lacZ* larvae were cultured in the presence of 100 ng/mL peptidoglycans from *Escherichia coli* (InvivoGen, San Diego, CA) and the compound at 25 °C for 12 h. The cultured individual larvae were sonicated with 200 μL reaction buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, and 1 mM $MgCl_2$) using an Ultrasonic Processor (Misonix, New York, NY). After centrifugation (10,000×g) at 4 °C for 10 min, supernatant was harvested, and β-galactosidase activity and total protein amount of supernatant were determined as previously described.[5a] β-Galactosidase activity was normalized to total protein amount.

3.12. Measurement of cytotoxicity and luciferase activity

Drosophila S2 cells and S2$^{att\text{-}luc}$ cells, harboring *luciferase* reporter gene driven by *attacin* promoter,[12] were cultured in Schneider's *Drosophila* medium (Gibco-BRL) supplemented with 10% FBS and 1% antibiotics/antimycotics at 25 °C. Cytotoxicity was measured using the colorimetric thiazoyl blue conversion assay using WST-8 solution (nacalai tesque) as described previously.[5b] To determine the effect of compounds on *attacin* expression via IMD pathway, S2$^{att\text{-}luc}$ cells were pre-treated with compounds for 1.5 h and stimulated by 100 ng/mL peptidoglycans. At 8 h after stimulation, cells were lysed with Glo-lysis buffer (Promega), and luciferase activities were measured by One-Glo (Promega).

Acknowledgements

We thank Dr. J.L. Imler for *Att-luc* reporter construct. This work was supported in part by Grant-in-Aid for Scientific Research (No. 23710247) from the Ministry of Education, Science, Sports and Culture of Japan, and the SUNBOR GRANT from the Suntory Institute for Bioorganic Research.

Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.tet.2013.02.088. These data include MOL files and InChiKeys of the most important compounds described in this article.

References and notes

1. Sleigh, M. A. *Protozoa and Other Protists*; Cambridge University Press: London, 1989.
2. Van der Auwera, G.; De Baere, R.; Van de Peer, Y.; De Rijk, P.; Van den Broeck, I.; De Wachter, R. *Mol. Biol. Evol.* **1995**, *12*, 671–678.
3. (a) William, F. *Mol. Plant Pathol.* **2008**, *9*, 385–402; (b) McDonald, B. A.; Linde, C. *Annu. Rev. Phytopathol.* **2002**, *40*, 349–379.
4. (a) McMorris, T. C.; Barksdale, A. W. *Nature* **1967**, *215*, 320–321; (b) Arsenault, G. P.; Biemann, K.; Barksdale, A. W.; McMorris, T. C. *J. Am. Chem. Soc.* **1968**, *90*, 5635–5636; (c) Green, D. M.; Edwards, J. A.; Barksdale, A. W.; McMorris, T. C. *Tetrahedron Lett.* **1971**, *27*, 1199–1203.

5. (a) Yajima, M.; Takada, M.; Takahashi, N.; Kikuchi, H.; Natori, S.; Oshima, Y.; Kurata, S. *Biochem. J.* **2003**, *371*, 205–210; (b) Sekiya, M.; Ueda, K.; Fujita, T.; Kitayama, M.; Kikuchi, H.; Oshima, Y.; Kurata, S. *Life Sci.* **2006**, *80*, 113–119.
6. Hoffmann, J. A.; Reichhart, J. M. *Nat. Immunol.* **2002**, *3*, 121–126.
7. Hultmark, D. *Curr. Opin. Immunol.* **2004**, *15*, 12–19.
8. (a) Sekiya, M.; Ueda, K.; Okazaki, K.; Kikuchi, H.; Kurata, S.; Oshima, Y. *Biochem. Pharmacol.* **2008**, *75*, 2165–2174; (b) Kikuchi, H.; Sekiya, M.; Katou, Y.; Ueda, K.; Kabeya, T.; Kurata, S.; Oshima, Y. *Org. Lett.* **2009**, *11*, 1693–1695; (c) Kikuchi, H.; Okazaki, K.; Sekiya, M.; Uryu, Y.; Ueda, K.; Katou, Y.; Kurata, S.; Oshima, Y. *Eur. J. Med. Chem.* **2011**, *46*, 1263–1273; (d) Sekiya, M.; Ueda, K.; Okazaki, K.; Terashima, J.; Katou, Y.; Kikuchi, H.; Kurata, S.; Oshima, Y. *Int. Immunopharmacol.* **2011**, *11*, 1497–1503; (e) Kikuchi, H.; Isobe, M.; Sekiya, M.; Abe, Y.; Hoshikawa, T.; Ueda, K.; Kurata, S.; Katou, Y.; Oshima, Y. *Org. Lett.* **2011**, *13*, 4624–4627; (f) Kikuchi, H.; Isobe, M.; Kurata, S.; Katou, Y.; Oshima, Y. *Tetrahedron* **2012**, *68*, 6218–6223.
9. Rychnovsky, S. D.; Yang, G.; Powers, J. P. *J. Org. Chem.* **1993**, *58*, 5251–5255.
10. Shiina, I.; Kobota, M.; Oshiumi, H.; Hashizume, M. *J. Org. Chem.* **2004**, *69*, 1822–1830.
11. Trost, B. M.; Balletire, J. L.; Godleski, S.; McDougal, P. G.; Balkovec, J. M. *J. Org. Chem.* **1986**, *51*, 2370–2374.
12. Goto, A.; Matsushita, K.; Gesellchen, V.; Chamy, L. E.; Kuttenkeuler, D.; Takeuchi, O.; Hoffmann, J. A.; Akira, S.; Boutros, M.; Reichhart, J.-M. *Nat. Immunol.* **2008**, *9*, 97–104.
13. Nakamura, S.; Iwami, J.; Matsuda, H.; Mizuno, S.; Yoshikawa, M. *Tetrahedron* **2009**, *65*, 2443–2450.
14. Luo, X.-D.; Wu, S.-H.; Ma, Y.-B.; Wu, D.-G. *Fitoterapia* **2000**, *71*, 668–672.
15. Gaulin, E.; Bottin, A.; Dumas, B. *Plant Signal. Behav.* **2010**, *5*, 258–260.