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ORIGINAL ARTICLE

Terpenoids produced by actinomycetes: isolation, structural elucidation and biosynthesis of new diterpenes, gifhornenolones A and B from *Verrucosispora gifhornensis* YM28-088

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New terpenoids named gifhornenolones A (1) and B (2) were isolated from the culture broth of *Verrucosispora gifhornensis* YM28-088, and their structures were established as hydroxylated isopimaradiene derivatives on the basis of extensive NMR and MS spectral analyses. In addition, a known sesquiterpene compound cyperusol C (3) was isolated. The absolute configuration of 1 was determined by nuclear Overhauser effect spectroscopy (NOESY) and CD spectra as 4*R*, 5*S*, 9*R*, 10*S*, 13*R*, and that of 2 was determined by NOESY experiments as 3*R*, 4*R*, 5*R*, 9*R*, 10*S*, 13*R*. Labeling experiments with [1-^{13}C]glucose and [*U*-$^{13}C_6$]glucose confirmed that the MEP (2-*C*-methyl-D-erythritol-4-phosphate) pathway was used for the biosynthesis of terpenoids in this organism. 1 showed potent inhibitory activity to the androgen receptor with an IC_{50} of 2.8 μg ml^{-1}.
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Keywords: androgen receptor binding inhibitor; biosynthesis; cyperusol C; gifhornenolone; terpenoid; *Verrucosispora gifhornensis*

INTRODUCTION

Marine microorganisms capable to produce secondary metabolites, marine actinomycetes, in particular, are an attractive resource for screening for bioactive compounds. Indeed, novel compounds exhibiting antitumor and/or antibacterial activity have been isolated from marine actinomycetes.[1] For instance, abyssomicin C isolated from *Verrucosispora* sp. showed antibacterial activity against Gram-positive bacteria including pathogenic *Staphylococcus aureus* strains as an inhibitor of the *para*-aminobenzoic acid biosynthesis pathway.[2–4] In addition, proximicin A produced by the same strain was reported to show antitumor activities.[5,6] Examples of bioactive metabolites isolated from the genus *Verrucosispora* were, however, quite limited presumably due to limited distribution of the genus *Verrucosispora* in the marine environment.

Previously, we carried out screening for terpenoids produced by actinomycetes and succeeded in isolation of several new derivatives such as oxaloterpins[7] and napyradiomycins.[8] In continuation of our work on the isolation of terpenoids of actinomycetes origin,[9] we attempted to obtain terpenoids from the genus *Verrucosispora*. Because terpenoids are lipophilic in most case, we analyzed crude solvent extracts of fermentation broths of several strains of this genus and succeeded in the isolation of two new diterpenoids named gifhornenolones A (**1**) and B (**2**) together with a known compound, cyperusol C (**3**) (Figure 1) from *Verrucosispora gifhornensis* YM28-088.

RESULTS AND DISCUSSION

Fermentation, extraction and isolation

V. gifhornensis YM28-088 that was isolated from an ascidian collected in Hiroshima, Japan, was cultured at 28 °C for 5 days by rotary shaking in 500 ml baffled Erlenmeyer flasks containing 100 ml of the culture medium. The broth was filtered and the broth filtrate was extracted with ethyl acetate (EtOAc). After removal of the solvent, we analyzed the residue extracted with EtOAc by thin layer chromatography (TLC) (*n*-hexane-EtOAc (1:1) or $CHCl_3$-MeOH (10:1), visualized by staining with vanillin-H_2SO_4). Spots appearing bright purple or violet on the TLC plate were selected as potential candidates for isolation. The mycelial cake was extracted with 60% aqueous acetone, and after removal of the solvent, the residual solution was extracted EtOAc and analyzed by TLC in the same manner as above.

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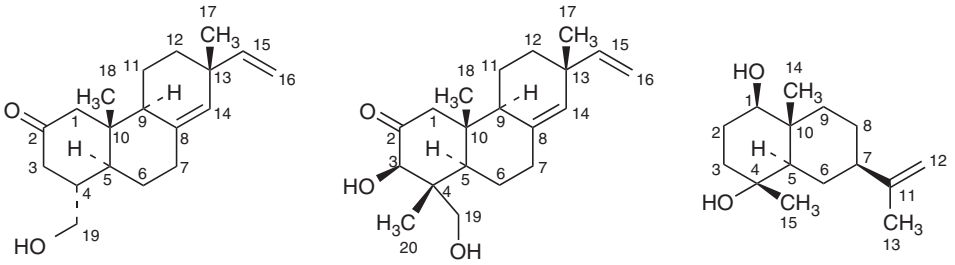
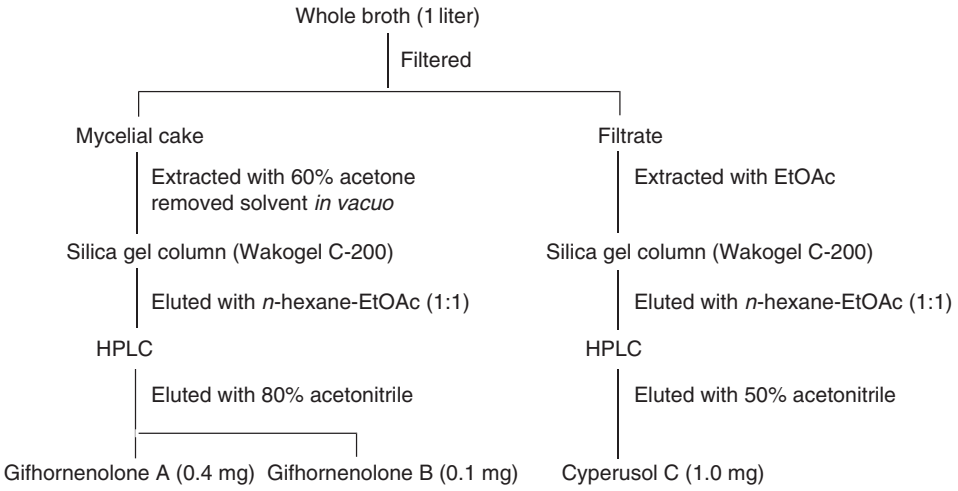


Figure 1 The structures of gifhornenolones A (**1**) and B (**2**), and cyperusol C (**3**).



Scheme 1 Isolation and purification of gifhornenolones A (**1**) and B (**2**), and cyperusol C (**3**).

Semi-preparative purification of these positive spots was carried out by Si-gel column chromatography and C-18 reverse-phase high-performance liquid chromatography (RP-HPLC) (Scheme 1). The purified samples thus obtained were analyzed by ^{1}H NMR and fractions showing that methyl signals at around δ1.0 were assumed to contain terpenoids[7,8], because almost all terpenoids possess several methyl groups. As a result of this screening, two fractions showing 2 or 3 methyl proton singlets were expected as terpenoids and subjected to detailed NMR analysis. NMR studies including COSY, HSQC and constant time-HMBC[10] (CT-HMBC) experiments, as well as HR-MS and IR, were used to determine the structures of the following terpenoids, gifhornenolones A (**1**) and B (**2**) from the mycelial cake extracts and cyperusol C (**3**) from the broth filtrate (Figure 1). (See Supplementary information for ^{1}H NMR, ^{13}C NMR, COSY, HSQC, CT-HMBC, nuclear Overhauser effect spectroscopy (NOESY), HR-MS, IR, LC-NMR of gifhornenolone A (**1**), B (**2**) and cyperusol C (**3**).)

Structure elucidation

Gifhornenolone A (**1**) was isolated as colorless needles. Its molecular formula was established as $C_{19}H_{28}O_2$ by HR-MS (*m/z* 289.2145 [M + H]$^+$, calcd 289.2168) indicating six degrees of unsaturation. The IR spectrum of **1** showed a ketone group (1701 cm^{-1}) and a hydroxyl group (3421 cm^{-1}) revealing the oxygen containing functionalities in **1**. The ^{13}C NMR and HSQC spectra confirmed the presence of 19 carbons (Table 1), including one ketone (δ_C 210.6 (C-2)), four olefinic carbons (δ_C 148.3 (C-15), 135.4 (C-8), 130.0 (C-14) and 110.6 (C-16)), one oxymethylene carbon (δ_C 63.9 (C-19)), three methine carbons (δ_C 47.8 (C-9), 45.0 (C-5) and 40.9 (C-4)), six methylene carbons (δ_C 53.4 (C-1), 44.2 (C-3), 34.7 (C-7), 34.1 (C-12), 25.2 (C-6) and 19.1 (C-11)), two singlet methyl groups (δ_C 26.2 (C-17) and 14.1 (C-18)) and two quaternary carbons (δ_C 42.8 (C-10) and 37.4 (C-13)). Further structural information on **1** was obtained by analyzing HSQC, CT-HMBC and COSY spectra.

The singlet methyl protons H-17 showed ^{1}H-^{13}C long-range couplings to C-12, C-13, C-14 and C-15, and the singlet methyl protons H-18 were coupled to C-1, C-5, C-9 and C-10 in the CT-HMBC spectrum. Partial structural information around the oxymethylene protons H-19 was obtained by its coupling to C-3, C-4 and C-5. The deshielded methylene protons H-1 and H-3 were connected to the carbonyl carbon by their couplings to C-2. The olefinic proton H-14 was coupled to C-7 and C-9 in addition to the C-8 sp^2 carbon. The exomethylene protons H-16 were coupled to an sp^2 carbon C-15 revealing the presence of a vinyl residue. This partial structure was corroborated by COSY correlations between H-15 and H-16. The quaternary sp^2 carbon C-8 was coupled to H-6, H-7, H-9 and H-11. In addition, COSY correlations were observed between H-19 and H-7 through H-4, H-5 and H-6, and between H-9 and H-12 through H-11. These results revealed the presence of an isopimaradiene skeleton in **1**.

The relative configuration of **1** was established by analysis of proton coupling constants and NOESY experiments as summarized in Figure 3. The stereochemistry at C-4 could not be determined directly by the splitting pattern of H-4 (1.77 p.p.m., m) due to its overlapping with H-6eq (1.77 p.p.m., m). However, H-5 at 1.74 p.p.m. (ddd, *J*=11.3, 11.3 and 3.5 Hz) gave satisfactory information. This proton was coupled with H-6eq (1.77 p.p.m., m) and H-6ax (1.17 p.p.m., dddd, *J*=4.5, 11.3, 13.5 and 13.5 Hz). Thus, the coupling constant between H-5 and H-6ax was determined to be 11.3 Hz suggesting H-5 is in axial orientation. The remaining coupling constant *J*=11.3 Hz

Table 1 ^{13}C- and ^{1}H-NMR spectral data for 1 and 2

		1			2		
C		δ (^{13}C)	δ (^{1}H)	Multiplicity[a]	δ (^{13}C)	δ (^{1}H)	Multiplicity[a]
1	CH_2	53.4	2.15[b]	d (13.1)	51.0	2.24	d (10.0)
	2.42[c]	dd (13.1, 2.3)		2.47	d (10.0)		
2	C	210.6			211.6		
3	CH_2	44.2	2.38[c]	ddd (14.0, 4.5, 2.3)	77.2[d]	4.35	m
			2.46[b]	dd (14.0,12.9)			
4	CH	40.9	1.77	m	49.2[e]		
5	CH	45.0	1.74	ddd (11.3, 11.3, 3.5)	45.2	2.05	dd (10.5, 2.5)
6	CH_2	25.2	1.17[b]	dddd (13.5,13.5, 11.3, 4.5)	21.9	1.41	m
			1.77[c]	m		1.64	m
7	CH_2	34.7	2.13[b]	ddd (13.5, 13.5, 2.0)	35.0	2.11[b]	m
			2.29[c]	ddd (13.5, 4.5, 2.0)		2.26[c]	ddd (12.0, 4.0, 1.5)
8	C	135.4			135.3		
9	CH	47.8	2.00	dd (7.6, 7.6)	50.2	2.01	dd (6.5, 6.5)
10	C	42.8			44.8		
11	CH_2	19.1	1.50, 1.54	m	18.8	1.50, 1.54	m
12	CH_2	34.1	1.40[b]	ddd (15.0, 11.0, 4.0)	34.1	1.37	ddd (14.0, 11.0, 3.5)
			1.43[c]	m		1.43	m
13	C	37.4			37.4		
14	CH	130.0	5.30	br s	130.1	5.24	br s
15	CH	148.3	5.76	dd (17.5, 10.0)	148.3	5.69	dd (15.0, 9.5)
16	CH_2	110.6	4.90	dd (10.0, 1.5)	110.6	4.84	dd (9.5, 1.5),
			4.92	de (17.5, 1.5)		4.85	dd (15.0, 1.5)
17	CH_3	26.2	1.03	s	26.1	0.98	s
18	CH_3	14.1	0.71	s	16.0	0.77	s
19	CH_2	63.9	3.61	dd (11.0, 2.5),	66.0	3.44	d (9.0)
			3.80	dd (11.0, 4.4)		3.49	d (9.0)
20	CH_3				12.9	0.55	s

NMR spectra were taken in $CDCl_3$.
[a]J in Hz.
[b]CH.
[c]C.
[d]Axial.
[e]Equatorial.

of H-5ax was assigned to the coupling with H-4 suggesting the stereochemistry at H-4 to be axial. This conclusion was also supported by the splitting pattern of H-3ax (12.9 and 14.0 Hz, the latter was assigned to geminal coupling to H-3eq).

NOE correlations were observed between H-4 and H-18, H-18 and H-11ax, and H-11ax and H-17 suggesting all these protons to be in axial orientation. In addition, H-5 showed an NOE to H-9.

The absolute configuration of **1** was clarified by measurement of the CD spectrum that exhibited a positive Cotton effect at 289 nm. Application of the octant rule to this positive Cotton effect clearly indicated that the axial methyl group C-18 is located in the upper left back octant. Consequently, the absolute configurations of the stereogenic centers of **1** were established as 4*R*, 5*S*, 9*R*, 10*S*, 13*R*.

The molecular formula of gifhornenolone B (**2**) was established as $C_{20}H_{30}O_3$ by HR-MS measurement (*m/z* 319.2270 $[M + H]^+$, calcd 319.2273). The ^{13}C NMR and HSQC spectra confirmed the presence of 20 carbons in **2**. Its NMR data were similar to those of **1** (Table 1) except for large downfield shifts of the oxymethine carbon (δ_C 77.2 (C-3)) and one quaternary carbon (δ_C 49.2 (C-4)) with appearance of an additional methyl carbon (δ_C 12.9 (C-20)). Further structural information on **2** was obtained by analyzing CT-HMBC and COSY spectra (Figure 2). Assuming the same stereochemistries for the gross structures of **1** and **2**, the stereochemistry at C-3 of **2** was concluded to be *R* by observation of NOE between H-3 and H-5 as shown in Figure 3.

Cyperusol C (**3**) was obtained as a colorless oil. Its NMR spectral data summarized in experimental section were in agreement with those reported for the known compound.[11] The absolute configuration of this compound was confirmed by NOESY experiments and negative optical rotatory dispersion (ORD) spectrum as summarized in Figure 3 as 3*R*, 4*R*, 5*R*, 9*R*, 10*S*, 13*R*. **3** was first purified from a plant *Cyperus longus*, but its isolation from microorganisms has never been reported.

Biosynthesis of isopentenyl diphosphate

Isopentenyl diphosphate, the starting material for the biosynthesis of terpenoids, is known to be biosynthesized mostly through the MEP (2-*C*-methyl-D-erythritol-4-phosphate) pathway[12] with some exceptions through the classical mevalonate pathway in actinomycetes. The latter examples include naphterpin,[13] terpentecin,[14] napyradiomycin[15] and BE-40644[16]. To examine which pathway was used in *V. gifhornensis* YM28-088, we carried out ^{13}C-labeling experiments with **1** and **3**.

Addition of [1-^{13}C]glucose to the fermentation broth of *V. gifhornensis* YM28-088 increased the signal intensities of C-2, C-6, C-11, C-14, C-16, C-17, C-18 and C-19 of **1** by approximately

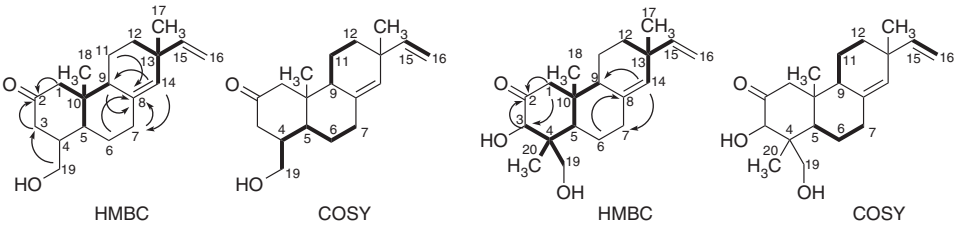


Figure 2 Important ^{1}H-^{13}C CT-HMBC correlations observed for gifhornenolones A (**1**) and B (**2**). Bold lines show HMBC correlations observed with methyl protons, or COSY correlations.

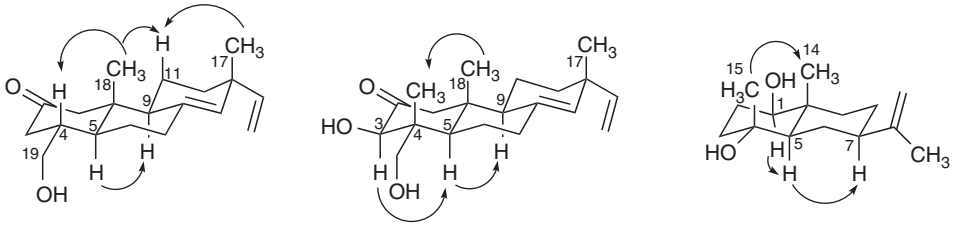


Figure 3 NOEs observed for gifhornenolones A (**1**) and B (**2**), and cyperusol C (**3**).

two times (Table 2). In **1** labeled with [*U*-$^{13}C_6$]glucose, eight pairs of ^{13}C-^{13}C couplings were observed between C-2 and C-3, C-4 and C-19, C-5 and C-6, C-8 and C-14, C-9 and C-11, C-10 and C-18, C-13 and C-17, and C-15 and C-16 leaving three enhanced singlet peaks due to C-1, C-7 and C-12. These labeling patterns of **1** were explained by operation of the MEP pathway in *V. gifhornensis* YM28-088 (Figure 4). In agreement with these results, no incorporation of sodium [1-^{13}C]acetate was observed with **1** (data not shown).

Assuming that **2** is a biosynthetic intermediate for **1** and that the carbon skeleton of gifhornenolone is formed by cyclization by chair–chair conformation of geranylgeranyl diphosphate, the biosynthetic scheme for **1** can be summarized as shown in Figure 5. At first, the β-methyl group of **2** is oxidized to carboxylic acid presumably by P450, and then the intermediate is converted to its 2-keto etc. by tautomerization to the 3-keto derivative that is very easily decarboxylated to give an enol derivative. After enolization, we converted the resultant 3-keto intermediate to 2-keto-3-hydroxy derivative by tautomerization. Finally, removal of the alcohol at C-3 may be carried out by dehydration and hydrogenation to give **1**.

In **3**, the signal intensities of C-2, C-6, C-8 and C-13 to 15 were enhanced by about 2.6-fold by the addition of [1-^{13}C]glucose. When labeled with [*U*-$^{13}C_6$]glucose, six pairs of ^{13}C-^{13}C couplings were detected between C-1 and C-2, C-4 and C-15, C-5 and C-6, C-7 and C-8, C-10 and C-14, and C-11 and C-13 with enriched singlet peaks of C-3, C-9 and C-12 (Table 2).

Table 2 Incorporation of [1-^{13}C]glucose and [U-$^{13}C_6$]glucose into 1 and 3

	1			*3*		
Position	δ_C	[1-^{13}C]glucose[a]	J_{C-C}[b]	δ_C	[1-^{13}C]glucose[a]	J_{C-C}[b]
1	53.4	1.0		79.5	1.0	36.5
2	210.6	2.4	38.5	28.6	2.7	36.7
3	44.2	1.1	38.3	40.9	1.0	
4	40.9	1.1	37.7	71.6	1.6	37.4
5	45.0	1.2	34.1	53.0	1.0	34.6
6	25.2	2.3	37.7	25.8	2.6	34.6
7	34.7	1.3		45.7	0.9	32.5
8	135.4	1.0	71.3	26.4	2.7	32.8
9	47.8	0.8	35.6	40.6	1.0	
10	42.8	1.0	36.3	39.0	1.5	36.0
11	19.1	2.4	35.0	150.3	1.5	41.3
12	34.1	1.0		108.4	1.1	
13	37.4	1.1	35.6	21.0	2.6	41.0
14	130.0	2.2	72.2	13.0	2.7	36.1
15	148.3	1.1	69.7	22.8	2.7	37.5
16	110.6	2.3	70.2			
17	26.2	2.0	35.5			
18	14.1	2.0	36.4			
19	63.9	1.9	38.5			

[a]Peak intensities were normalized to C-1 carbon in both the compounds.
[b]Carbon–carbon coupling constants observed with samples labeled by [$^{13}C_6$]glucose.

Biological activity

Because isopimarane-type diterpenes have structural similarity to steroidal compounds such as dihydrotestosterone (DHT) and other known androgen antagonists, we expected that **1** might show androgen antagonist activity. **1** showed inhibitory activity against binding of DHT to AR (androgen receptor) with an IC_{50} of 9.7 μM ml^{-1} (*in vitro* binding assay, using [^{3}H]-DHT and recombinant AR).

EXPERIMENTAL SECTION

General experimental procedures

Both 1D ^{1}H and ^{13}C NMR spectra were recorded on a JEOL Alpha 400 NMR spectrometer (JEOL, Akishima, Tokyo, Japan, operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C) or a Varian Inova 500 (Varian, Palo Alto, CA, USA,

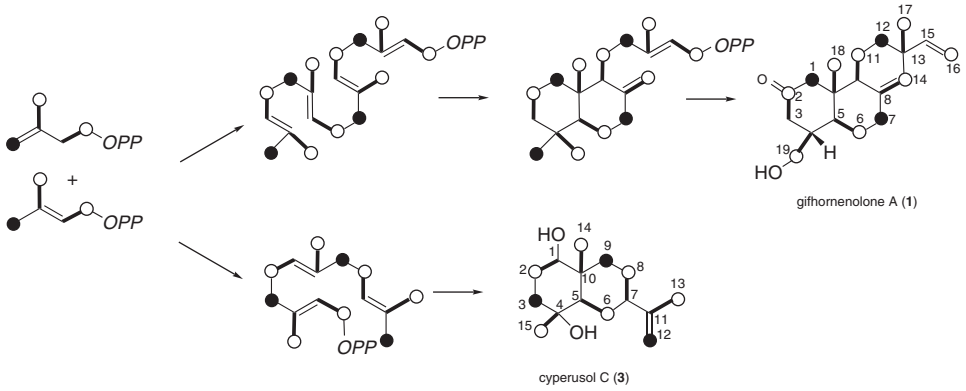


Figure 4 Incorporation patterns of isopentenyl diphosphate (IPP) from [1-^{13}C]glucose and [*U*-$^{13}C_6$]glucose into gifhornenolones A (**1**) and cyperusol C (**3**). Open circles show carbons derived from [1-^{13}C]glucose through the MEP pathway. Closed circles indicate carbons derived from C-3 of pyruvic acid. Bold lines show ^{13}C-^{13}C couplings observed with a sample enriched by [*U*-$^{13}C_6$]glucose.

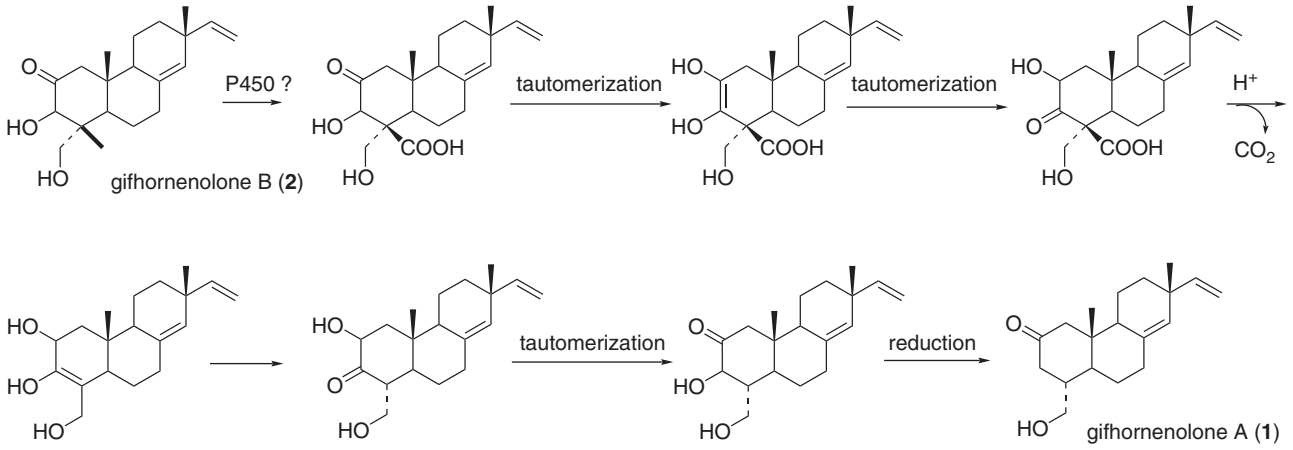


Figure 5 Hypothetical biosynthetic pathway from gifhornenolone B (**2**) to gifhornenolone A (**1**).

operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C). Two-dimensional ^{1}H-^{1}H COSY, NOESY, ^{1}H-^{13}C HSQC, CT-HMBC spectra were recorded on a Varian Inova 500 or JEOL ECA 600 NMR spectrometer. Samples were dissolved in $CDCl_3$ and the solvent peak was used as an internal standard (δ_H 7.24 and δ_C 77.0). HR-ESI-MS data were recorded on a Waters LCT-Premier XE mass spectrometer (Waters, Milford, MA, USA). IR spectra were obtained in KBr with a Shimadzu 8300 FTIR spectrometer (Shimadzu, Kyoto, Japan). Optical rotations were measured on a HORIBA SEPA-300 polarimeter (HORIBA, Kyoto, Japan). CD spectra were recorded on a Jasco J-720WI spectropolarimeter with ORDM-306 attachment (Jasco, Tokyo, Japan). ORD spectra were obtained with a Jasco J-720WI (cylindrical quartz cell ϕ 3.5×100 mm). HPLC purifications were carried out using a SENSHU PAK PEGASIL ODS column (20×250 mm) equipped with a Hitachi High Technologies L-2450 diode array detector (Hitachi, Ibaraki, Japan). Wako Wakogel C-200 was used for Si gel column chromatography (Wako, Osaka, Japan); Si gel 60 F_{254} plastic-backed sheets were used for TLC analysis.

Cultivation of *V. gifhornensis* YM28-088

V. gifhornensis YM28-088 was inoculated with the mycelia of the strain grown on an agar slant into 15 ml test tubes containing 5 ml of a preliminary seed medium consisting of soluble starch 1.0%, polypeptone 1.0%, molasses 1.0% and meat extract 1.0% (pH 7.2 before sterilization), and was cultured at 28 °C for 7 days on a rotary shaker at 170 r.p.m. Aliquot (1 ml) of the seed culture was inoculated into each of 500 ml baffled Erlenmeyer flasks containing 100 ml of the medium consisting of starch 2.0%, polypeptone 0.5%, meat extract 0.5%, dry yeast 0.3% and $CaCO_3$ 0.3% (pH 7.0 before sterilization). The microorganism was cultured at 28 °C for 5 days. This strain is maintained at Marine Biosciences, Kamaishi Research Laboratory, Kitasato University, Heita, Kamaishi, Iwate, Japan.

Purification of gifhornenolones A (1) and B (2)

The fermentation broth (1 liter) was separated into mycelial cake and filtrate by suction filtration. The supernatant was extracted with an equal amount of EtOAc, the organic layer was dried over anhydrous Na_2SO_4 and the solvent was removed under reduced pressure. After mycelial cake was submerged in 60% acetone-water, we removed the solvent under reduced pressure, and extracted the aqueous residue with an equal amount of EtOAc.

The EtOAc extract from the mycelia cake was subjected to Si gel column chromatography (*n*-hexane-EtOAc, 1:1). The eluted fractions were analyzed by color reaction with vanillin-H_2SO_4 on Si gel TLC ($CHCl_3$-MeOH, 10:1). Fractions containing **1** and **2** (Rf 0.4) were combined and further purified by ODS HPLC (20×250 mm; SENSHU PAK PEGASIL ODS) with a PDA detector eluted with CH_3CN in H_2O (80%) at a flow rate of 14 ml min^{-1} to yield **1** (0.4 mg l^{-1}, 9.7 min) and **2** (0.1 mg l^{-1}, 10.1 min).

Gifhornenolone A (**1**). Colorless needles, m.p. 94–95 °C (crystallized from AcOEt—*n*-hexane), $[\alpha]_D^{25}$ +6.6° (*c* 0.18, $CHCl_3$), CD (*c*=1.7, MeOH): Δε=−14.35 (210 nm, neg. max.) Δε=+2.0 (289 nm, pos. max.), IR (KBr) 1701, 3422 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$), see Table 1; HR-MS *m/z* 289.2145 (calcd for $C_{19}H_{28}O_2$, [M + H]$^+$ 289.2168).

*Gifhornenolone B (**2**).* Colorless oil, ^{1}H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$), see Table 1; HR-MS *m/z* 319.2270 (calcd for $C_{19}H_{28}O_2$, $[M + H]^+$ 319.2273).

*Purification of cyperusol C (**3**).* The EtOAc extract from the filtrate of *V. gifhornensis* YM28-088 was subjected to Si gel column chromatography (*n*-hexane-EtOAc, 1:2). **3** was visualized by color reaction with vanillin-H_2SO_4 on Si gel TLC (*n*-hexane-EtOAc, 1:1). Fractions containing cyperusol C (Rf 0.2) were combined and further purified by ODS HPLC (20×250 mm; SENSHU PAK PEGASIL ODS) with a PDA detector eluted with CH_3CN in H_2O (50%) at a flow rate of 14 ml min^{-1} to yield cyperusol C (1.0 mg l^{-1}, 8.8 min).

*Cyperusol C (**3**).* Colorless oil, ^{1}H NMR data, δ_H 0.89, 1.12, 1.74 (3H each, s, H_3-14, 15, 13), 1.14 (1H, ddd, *J*=4.0, 13.0, 13.5 Hz, H-9), 1.88 (1H, ddd, *J*=3.5, 3.5, 13.5 Hz, H-9), 1.26, 1.84 (1H each, both m, H-6), 1.28 (1H, m, H-5), 1.38 (1H, dddd, *J*=3.5, 13.0, 13.5, 17.0 Hz, H-8), 1.52 (1H, ddd, *J*=3.5, 12.0, 13.5 Hz, H-3), 1.62 (1H, m, H-8), 1.60, 1.72 (1H each, both m, H-2), 1.80 (1H, ddd, *J*=3.0, 3.5, 12.0 Hz, H-3), 1.92 (1H, m, H-7), 3.34 (1H, dd, *J*=4.4, 11.2 Hz, H-1), 4.70 (2H, m, H_2-12); ^{13}C NMR data, δ_C 79.5 (C-1), 28.6 (C-2), 40.9 (C-3), 71.6 (C-4), 53.0 (C-5), 25.8 (C-6), 45.7 (C-7), 26.4 (C-8), 40.6 (C-9), 39.0 (C-10), 108.4 (C-11), 150.3 (C-12), 21.0 (C-13), 13.0 (C-14), 22.8 (C-15). HR-MS *m/z* 239.2010 (calcd for $C_{19}H_{28}O_2$, $[M + H]^+$ 239.2011).

Biosynthetic experiment

[1-^{13}C]Glucose (1 mg ml^{-1}), sodium [1-^{13}C]acetate (1 mg ml^{-1}) and [*U*-$^{13}C_6$] glucose (1 mg ml^{-1}) were added to the medium 38 h after initiation of the fermentation at separate experiments. Production of **1** and **3** started at about 38 h after cultivation. After 5 days, we purified the labeled samples of **1** and **3** as above, and then subjected them to ^{13}C-NMR NMR spectral analysis.

Biological activity

In vitro AR binding activity was assayed as previously reported with some modifications.[17,18] In brief, 50 μg ml^{-1} recombinant androgen receptor C-termini protein, 2 nM [^{3}H]-DHT and a test compound were mixed in a binding buffer consisting of 50 mM Tris-HCl (pH 7.4), 800 mM NaCl, 10% glycerol, 1 mg ml^{-1} bovine serum albumin and 2 mM DTT to give a 100 μl mixture solution. The mixture was incubated at 4 °C for 3 h, and BioGel HT (Bio-Rad Laboratories, Hercules, CA, USA) was added to the solution and further incubated on ice for 15 min. [^{3}H]-DHT-bound BioGel HT was washed with washing buffer (40 mM Tris-HCl (pH 7.6), 100 mM KCl, 1 mM EDTA and 1 mM EGTA) three times, and its radioactivity was measured by a liquid scintillation counter.

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