ORIGINAL ARTICLE

Structures and absolute stereochemistry of dinapinones A1 and A2, inhibitors of triacylglycerol synthesis, produced by *penicillium pinophilum* FKI-3864

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During our screening program for microbial inhibitors of triacylglycerol synthesis in mammalian cells, four structurally related new compounds, dinapinones A1 (1) and A2 (2) and monapinones A (3) and B (4), were isolated from the culture broth of *Penicillium pinophilum* FKI-3864. Compounds 3 and 4 were produced by the fungus only when fermented in seawater-supplemented medium. The structures of 1 to 4 were elucidated by spectroscopic studies including various NMR experiments. Compounds 1 and 2 were atropisomers consisting of two monomers with the same planar structure of dihydronaphthopyranone as 3. The absolute stereochemistry of 3 was elucidated by NOE experiment and circular dichroism spectra. Furthermore, the stereochemistry of 1 and 2 was elucidated by *in vitro* conversion from the structure-defined 3 to its dimers 1 and 2.

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INTRODUCTION

Triacylglycerol (TG) synthesis is important in many metabolic processes in mammals, including lactation, energy storage in fat and muscle, fat absorption in the intestine and the assembly of lipoprotein particles in the liver and small intestine; however, excess accumulation of TG in certain organs and tissues causes fatty liver, obesity and hypertriglyceridemia.^{2–4} Recently, much attention has been paid to lifestyle-related metabolic syndrome as a high-risk condition leading to severe diseases such as atherosclerosis, hypertension and diabetes. For example, accumulation of TG in the liver has been thought to trigger the inflammation, apoptosis, and fibrosis that characterize the progression of hepatic steatosis to nonalcoholic steatohepatitis and cirrhosis.⁵ Against this background, we established a cell-based assay system using Raji cells⁶ and Chinese hamster ovary-K1 (CHO-K1) cells⁷ to screen microbial metabolites for inhibitors of TG synthesis. Through our continuous screening efforts, we recently discovered a series of new dihydronaphthopyranone-containing compounds named dinapinones A1 (1) and A2 (2) (Figure 1) and monapinones A (3) and B (4) in the culture of *Penicillium pinophilum* FKI-3864. Study of the culture conditions indicated that monapinones were produced by the fungus, when the strain was fermented in the original dinapinone production medium supplemented with seawater.^{7,8} In this study, we demonstrated that 1

and 2 are atropisomers consisting of dimers of 3 and that the absolute stereochemistry of 1 and 2 is elucidated by conversion of structure-defined 3 to 1 and 2.

RESULTS

Structures of dinapinones A1 (1) and A2 (2)

Compound 1 showed a molecular ion peak at m/z 835 ($M+H$)⁺ in FAB/MS, and the molecular formula C₄₆H₅₈O₁₄ was assigned on the basis of its HRFAB/MS [m/z 835.3923 ($M+H$)⁺, Δ +1.8 mmu], indicating 18 degrees of unsaturation. The ¹H and ¹³C NMR spectra of 1 (Tables 1 and 2) showed 27 protons and 23 carbons signals on the basis of 2D NMR correlations of 1, suggesting that 1 forms a symmetric dimer structure. The multiplicity of the carbon signals was classified into one methyl carbon, one oxygenated methyl carbon, seven *sp*³ methylene carbons, two *sp*² methine carbons, three *sp*³ oxygenated methine carbons, five *sp*² quaternary carbons, three *sp*² oxygenated quaternary carbons and one carboxyl carbon by analysis of DEPT and HMQC data. The connectivity of proton and carbon atoms was established by HMQC (Tables 1 and 2). As shown by the bold lines in Figure 2, the partial structure I was elucidated by ¹H-¹H COSY and HOHAHA spectra. ¹H-¹³C long-range couplings of ²*J* and ³*J* in the HMBC are also shown in Figure 2, leading to the following results: cross peaks from the *sp*³ oxymethine proton H-3 (δ 4.88) to

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The name of dinapinones and monapinones-producing fungus *Penicillium pinophilum* has been changed to *Talaromyces pinophilus* in 2011.¹

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Figure 1 Structures of 1 to 6.

Table 1 ^1H NMR chemical shifts of dinapinones A1 (1) and A2 (2) and monapinones A (3) and B (4) in CDCl_3

Position	1			2			3			4		
	δ_{H}	J (Hz)		δ_{H}	J (Hz)		J (Hz)			δ_{H}	J (Hz)	
3	4.88	1H	dddd	4.2, 6.0, 7.4, 10.2	4.88	1H	dddd	4.2, 6.0, 7.4, 10.2	4.87	1H	m	
4	3.07	1H	dd	10.2, 16.2	3.07	1H	dd	10.2, 16.2	3.04	1H	dd	9.5, 16.0
	3.13	1H	dd	4.2, 16.2	3.13	1H	dd	4.2, 16.2	3.10	1H	dd	4.0, 16.0
5	6.97	1H	s		6.97	1H	s		6.90	1H	s	
6	6.71	1H	s		6.71	1H	s		6.57	1H	d	2.5
8									6.54	1H	d	2.5
									3.89	3H	s	
11	3.84	3H	s		3.84	3H	s		2.14	1H	ddd	7.0, 8.0, 14.2
12	1.89	1H	ddd	4.0, 6.0, 14.0	1.88	1H	ddd	3.8, 6.2, 13.7	1.88	1H	ddd	4.0, 6.0, 14.2
	2.14	1H	ddd	7.4, 8.0, 14.0	2.14	1H	ddd	7.0, 8.3, 13.7	1.90	1H	ddd	3.5, 6.0, 14.5
13	4.22	1H	dddd	2.8, 4.0, 8.0, 9.5	4.20	1H	dddd	2.8, 3.8, 8.3, 9.5	4.20	1H	m	
14	1.62	1H	ddd	9.5, 9.5, 14.4	1.62	1H	ddd	9.5, 9.5, 14.7	1.61	1H	ddd	10.0, 10.0, 14.5
	1.68	1H	ddd	2.8, 2.8, 14.4	1.67	1H	ddd	2.8, 2.8, 14.7	1.66	1H	ddd	2.2, 2.5, 14.5
15	3.93	1H	m		3.92	1H	m		3.92	1H	m	
16	1.49	2H	m		1.49	2H	m		1.49	2H	m	
17	1.30	2H	m		1.30	2H	m		1.31	2H	m	
18	1.30	2H	m		1.30	2H	m		1.31	2H	m	
19	1.30	2H	m		1.30	2H	m		1.31	2H	m	
20	0.90	3H	t	6.6	0.90	3H	t	6.6	0.90	3H	t	6.7
OH-9	9.68	1H	s		9.67	1H	s		9.46	1H	s	
OH-10	13.70	1H	s		13.70	1H	s		13.68	1H	s	
										13.67	1H	s

Chemical shifts are shown in δ values (p.p.m.) relative to CDCl_3 at 7.26 p.p.m.

the carboxyl carbon C-1 (δ 171.3) and the sp^2 quaternary carbon C-4a (δ 132.73), from the sp^3 methylene proton H₂-4 (δ 3.07, 3.13) to the sp^2 quaternary carbons C-4a, C-5 (δ 116.26) and C-10a (δ 99.3) and

from the sp^2 methine proton H-5 (δ 6.97) to the sp^3 methylene carbon C-4 (δ 32.98) and the sp^2 quaternary carbons C-4a, C-5a (δ 140.1), C-9a (δ 108.2) and C-10a indicated the presence of an

isochroman-1-one moiety containing the partial structure I. The cross peak from the sp^2 methine proton H-6 (δ 6.71) to the sp^2 oxygenated quaternary carbon C-7 (δ 161.5), the sp^2 quaternary carbon C-8 (δ 108.4) and C-9a, from 9-OH (δ 9.68) to C-8, C-9 and C-9a, and from the oxygenated methyl proton H₃-11 (δ 3.84) to C-7 indicated the presence of a 3-methoxy-2,5,6-trisubstituted phenol moiety. This moiety was connected to the isochroman-1-one moiety by the 4J cross peak from H-5 to C-1 and C-9, from H-6 to C-10 and from 9-OH to C-7 in HMBC experiments, indicating the presence of a dihydronaphthopyranone (Figure 2). Further, the chemical shifts and half of the molecular formula ($C_{23}H_{29}O_7$) of **1** indicated that three hydroxyl groups are attached to C-10, C-13 (δ 69.60) and C-15 (δ 73.36). Thus, half of the molecule of **1** (partial structure II) was elucidated as shown in Figure 2. Furthermore, ^{13}C NMR chemical shift data of the carbonyl and aromatic carbons in **1** were almost identical to those in (*R*)-semiovioxanthin (**5**), except that the methine C-8 signal (δ 99.1) in **5**^{9,10} was replaced by a quaternary carbon signal (C-8; δ 108.4) in **1**; therefore, **1** should have an 8,8'-linkage between

the two halves of the molecule (partial structure II). From the observations described above, the planar structure of **1** was elucidated and fulfilled the molecular formula and the degrees of unsaturation.

The 1H and ^{13}C NMR spectra (Tables 1 and 2) and 2D NMR (1H - 1H COSY, HMQC and HMBC) data of **2** resembled those of **1**. Accordingly, the planar structure of **2** was also elucidated to be the same as **1**.

Stereochemistry of dinapinones A1(**1**) and A2 (**2**)

Compounds **1** and **2** have six chiral carbons and one axial configuration. The relative stereochemistry of the partial structure II (the α,β -unsaturated δ -lactone having a dihydro alkyl chain) at C-3 in **1** was elucidated by analysis of the dihedral angles and the 1H NMR coupling constants. The large value of J_{3-4a} (10.2 Hz) indicated that the two protons are in *trans* diaxial orientation (Figure 3). In addition, an NOE was observed between 3-H (δ 4.88) and 4-H_b (δ 3.07) in ROESY experiments, showing that 3β configuration was assigned for **1** (Figure 4). Furthermore, the relative stereochemistry of the partial structure II of **1** was confirmed by analysis of 1H NMR coupling constants (Figure 3) and ROESY experiments (Figure 4). NOEs were observed between 4-H_a and 12-H₂ (δ 2.14, 1.89) and between 12-H₂ and 14-H₂ (δ 1.68, 1.62), indicating that they are all oriented to the same side of the α,β -unsaturated δ -lactone plane. On the other hand, NOEs were observed between 4-H_b and 3-H/13-H (δ 4.22), between 3-H and 13-H and between 13-H and 15-H (δ 3.93), indicating that they are all oriented to the opposite side of the α,β -unsaturated δ -lactone plane. Furthermore, the relative stereochemistry of the 13,15-dihydroxy groups was confirmed by NMR analysis of the acetone derivative of **1** as also applied to the stereochemistry elucidation in polyene macrolide and polyketide compounds,^{11,12} in which NOE was investigated between the acetone methyl protons and the protons geminal to the oxygen atoms. As illustrated in Figure 5, C-21 and C-22 of the acetone derivative prepared from **1** were assigned to δ 29.7 (1H; δ 1.39) and δ 19.8 p.p.m. (1H; δ 1.47), respectively, and NOEs were observed between axial 21-H₃ and 13-H/15-H, indicating that the two hydroxy groups are in *syn* configuration. Thus, the relative stereochemistry of **1** was presumed to be $3S^*$, $13R^*$ and $15R^*$.

The 1H NMR coupling constants and ROESY experimental data of **2** resembled those of **1** (Table 1). Accordingly, the relative stereochemistry of **2** was determined to be $3S^*$, $13R^*$ and $15R^*$. Thus, **1** and **2** are dimers of the partial structure II with the same relative stereochemistry, suggesting that they are atropisomers.

To elucidate the absolute axial configuration, the CD data of **1** and **2** were compared with those reported for (*P*)- and (*M*)-vioxanthin (**5**).¹²⁻¹⁴ Compound **1** exhibited a first negative cotton effect at

Table 2 ^{13}C NMR chemical shifts of dinapinones A1 (**1**) and A2 (**2**) and monapinones A (**3**) and B (**4**) in $CDCl_3$

Position	1		2		3		4	
1	171.3	s	171.3	s	169.7	s	171.2	s
3	78.18	d	78.13	d	78.1	d	78.8	d
4	32.98	t	32.84	t	33.6	t	33.0	t
4a	132.73	s	132.70	s	132.1	s	132.9	s
5	116.26	d	116.28	d	115.5	d	116.1	d
5a	140.1	s	140.1	s	139.5	s	140.5	s
6	98.1	d	98.1	d	99.1	d	99.5	d
7	161.5	s	161.5	s	161.6	s	163.0	s
8	108.4	s	108.4	s	101.2	d	101.6	d
9	155.4	s	155.4	s	157.2	s	158.5	s
9a	108.2	s	108.2	s	107.8	s	108.3	s
10	162.9	s	162.82	s	161.3	s	162.7	s
10a	99.3	s	99.3	s	98.9	s	99.3	s
11	55.9	d	55.9	d	55.8	d	55.5	d
12	42.25	t	42.14	t	42.8	t	41.8	t
13	69.60	d	69.54	d	69.6	d	69.1	d
14	42.91	t	42.91	t	43.4	t	37.7	t
15	73.36	d	73.36	d	73.4	d	25.1	d
16	38.38	t	38.40	t	39.0	t	31.7	t
17	24.9	t	24.9	t	25.8	t	22.6	t
18	31.7	t	31.7	t	32.4	t	14.0	q
19	22.6	t	22.6	t	23.4	t		
20	14.0	q	14.0	q	15.0	q		

Chemical shifts are shown in δ values (p.p.m.) relative to $CDCl_3$ at 77.0 p.p.m.

Figure 2 Structure elucidation of **1** by 1H - 1H COSY, HOHAHA and HMBC experiments.

Figure 3 Coupling constants ($^2J_{HH}$) and the dihedral angles of partial structure II of **1**.

Figure 4 ROESY stereochemistry of partial structure II of **1**. A full color version of this figure is available at the *Journal of Antibiotics* online.

Figure 5 Relative configuration of acetonide derivative of **1**.

280 nm and second positive cotton effect at 251 nm in the CD spectrum. On the other hand, **2** exhibited a first positive cotton effect at around 278 nm and second negative cotton effect at around 255 nm in the CD spectrum. These spectra conformed to *M*- and *P*-vioxanthin, respectively. Accordingly, the respective absolute axis configurations of **1** and **2** were elucidated to be *M* and *P*.

Structures of monapinones A (**3**) and B (**4**)

Compound **3** had a molecular ion peak at m/z 417 ($M-H$)[−] in ESI-TOF-MS, and the molecular formula $C_{23}H_{30}O_7$ in HRESI-TOF-MS [m/z 417.1925 ($M-H$)[−], $\Delta +0.2$ mmu], requiring 9 degrees of unsaturation. UV absorption indicated the presence of a

naphthopyranone skeleton.⁴ 1H and ^{13}C NMR spectra of **3** showed 28 proton and 23 carbon signals, and the multiplicity of carbon signals was classified into one methyl carbon, seven methylene carbons, three oxygenated methine carbons, three sp^2 methine carbons, one oxygenated methyl carbon, four sp^2 quaternary carbons, three oxygenated sp^2 quaternary carbons, and one carbonyl carbon by analysis of the HSQC of spectra. Taking the molecular formula into consideration, the presence of two hydroxy protons was suggested. The connectivity of proton and carbon atoms was established by the HSQC spectrum (Tables 1 and 2). Interestingly, the 1H and ^{13}C -NMR spectra of **3** were almost identical to those of **1** except for the presence of one aromatic methine signal (δ 6.54 and δ 101.2) in **3**. These data accounted for about half of the molecular formula, suggesting that **3** was the monomer structure of **1**. To confirm the structure, various 2D NMR experiments were carried out. As shown in Figure 6a, analyses of 1H - 1H COSY and long-range couplings (2J and 3J) in the HMBC spectrum revealed the presence of the same long alkyl chain and the same chromophore as observed in **1**. The different point was that newly observed proton (δ 6.54) and carbon signals (δ 101.2) in **3** were assigned at the 8 position, because cross peaks from H-6 (δ 6.57) to C-8 (δ 101.2), from H-8 (δ 6.54) to C-6 (δ 99.1), C-7 (δ 161.6) and C-9 (δ 157.2) and from OH-9 (δ 9.46) to C-8 and C-9 were observed. A small coupling constant value (2.5 Hz) between H-6 and H-8 also indicated that the two aromatic protons were oriented in a meta-position. Taken together, the planar structure of **3** was concluded to be the half part of **1** (Figure 6a). The structure satisfied the degree of unsaturation and the molecular formula.

Compound **4** had physicochemical properties similar to **3**, indicating that they share a common naphthopyranone skeleton with a long alkyl chain. Additionally, HRESI-TOF-MS [m/z 373.1650 ($M-H$)[−], $\Delta -0.1$ mmu] of **4** indicated that the molecular formula $C_{21}H_{26}O_6$ was smaller by C_2H_4O than **4**. Analyses of 1H - 1H COSY revealed the partial structure I (bold lines in Figure 6b), which had a shorter alkyl chain by C_2H_2 than that of **1**. In addition, the chemical shift of C-13 (δ 69.1) showed the presence of a hydroxy group. Connection to the partial structure I was also confirmed by observation of the cross peaks observed from H-3 (δ 4.85) to C-4a (δ 132.9), from H2-4 (δ 3.08) to C-4a, C-5 (δ 116.1) and C-10a (δ 93.3) and from H-5 (δ 6.89) to C-4 (δ 33.0), C-4a and C-10a in HMBC experiments (Figure 6b). Taken together, the structure of **4** was elucidated as shown in Figure 1. The structure satisfied the degree of unsaturation and the molecular formula.

Absolute Stereochemistries of monapinones A (**3**) and B (**4**)

Compound **3** has three chiral carbons at C-3, C-13 and C-15. As shown in Figure 4, the conformations of C-4/C-3, C-3/C-12, C-12/C-13, C-13/C-14 and C-14/C-15 were determined by the coupling constants in 1H NMR and ROESY experiments. The coupling constants of **3** were almost identical to those of **1**, indicating that the relative stereochemistry of **3** could be elucidated to be $3S^*,13R^*,15R^*$. Furthermore, the CD spectrum of **3** was investigated to define the absolute configuration at C-3. Compound **3** exhibited a first negative cotton effect at 266 nm and a second positive cotton effect at 238 nm in the CD spectrum. Comparison of the data with those reported for (*S*)- and (*R*)-semiovioxanthins² indicated that the absolute stereochemistry at C-3 in **3** was elucidated to be *3S*. Accordingly, the total absolute stereochemistry of **3** was concluded to be $3S,13R,15R$. The absolute stereochemistry of **4** was also determined to be $3S,13R$ because of the similarity of the coupling constants in 1H NMR and ROESY experiments of **3**.

Figure 6 Structure elucidation of **3** (a) and **4** (b) by ^1H - ^1H COZY and HMBC experiments.

Elucidation of absolute stereochemistry of dinapinones A (1) and B (2) by conversion of monapinone A (3)

To determine the absolute stereochemistry, we attempted to derivatize **1** and **2** to *p*-bromo-benzoyl and α -methoxy- α -(trifluoromethyl)phenylacetic ester analogs, but the derivatization was not successful. Therefore, we tried a different method to convert structure-defined **3** to **1** and **2**. Several groups have reported the conversion of dihydronaphthopyranone-type monomers to dimers, but there have been debates on whether the reaction proceeds chemically or enzymatically. We tested two conditions: A) **3** was incubated with hydrated ferric chloride ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), or B) with lysate prepared from *P. pinophilum* FKI-3864 at 27°C for 20 min, and the products were analyzed by HPLC using a C30 column. As shown in Figure 7a, **1** and **2** were selectively produced under condition B. The amount of **1** and **2** time-dependently increased in a ratio of 2:3 with a concomitant decrease of **3** (Figure 7b). On the other hand, many peaks were observed under condition A, but peaks corresponding to **1** and **2** were not detected, at least up to 25 min. These data strongly suggested that **1** and **2** were enzymatically produced from the monomer **3**. Accordingly, the absolute stereochemistry of **1** and **2** was proved to be *M*, 3*S*, 13*R*, 15*R* and *P*, 3*S*, 13*R*, 15*R*, respectively.

DISCUSSION

In the course of screening for microbial inhibitors of TG synthesis in a cell-based assay using CHO-K1 cells, seven structurally related dihydronaphthopyranones, dinapinones A1 and A2 and monapinones A to E, were isolated from the culture broth of *P. pinophilum* FKI-3864.^{7,8} From a study of the culture conditions, monapinones were found to be produced only in a dinapinone-producing medium supplemented with seawater.⁸

As described in this study, the structures of **1** to **4** were elucidated by various spectral analyses. Compounds **1** and **2** with the same planar structure were found to be dimers of **3**. From comparison of CD spectra with those of vioxanthins,^{13,14} **1** and **2** were atropisomers with the same relative stereochemistry of the acyl side chains in their structures; however, the complete absolute stereochemistry was not elucidated by spectral analyses. On the other hand, the structure of **3** including the absolute stereochemistry was defined to be 3*S*,13*R*,15*R* by the comparison of CD spectra of (*S*)- and (*R*)-semivioxanthins.²

To elucidate the absolute stereochemistry of **1** and **2**, we tried to convert absolute stereochemistry-defined monomer **3** to its dimers **1** and **2**. Several groups reported dimerization experiments from aromatic monomers. Bode *et al.*¹⁵ carried out feeding of absolute structure-defined monomer *R*-semivioxanthin (**5**) in vioxanthin-producing *Penicillium citreoviride*. They found that **5** was converted

Figure 7 Production of dimers of **1** and **2** by incubation of monomer **3** with cell lysates prepared from *P. pinophilum* FKI-3864 and with $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$. (a) Time course of conversion of **3** to **1** and **2** under the enzymatic dimerization. ● starting material of **3**, ▲ **1** and ■ **2**. (b) HPLC analysis of converted products under the chemical and enzymatic dimerization using a C30 column.

to its dimer *R,R*-vioxanthin ((*R,R*)-**6**) by regio- and stereoselective intermolecular oxidative phenol coupling, and finally elucidated the absolute axis configuration as (*P,R,R*)-**6** by comparison with the synthesized (*P,R,R*)-**6** and (*M,R,R*)-**6** in CD spectra.¹⁵ These findings indicated that dimerization is proceeded by enzymatic reactions. On the other hand, Drochner *et al.* tried to convert monomer orsellinate (methyl 2-hydroxy-4-methoxy-6-methyl-benzoate, **7**) to its dimers under chemical conditions.¹⁶ According to the reaction of oxidative phenolic coupling, **7** was treated with FeCl_3 adsorbed on silica gel, yielding 3,3'-, 5,5'- and 3,5'-dimers by 17%, 33 and 30% yield, respectively.¹⁶ Zeek *et al.* also attempted oxidative phenol coupling of **5** using $\text{Cu}(\text{Ac})_2$, Ag_2O , $(\text{NH}_4)_2[\text{Ce}(\text{NO}_3)_6]$, 2,3-dichloro-5,6-dicyano-*p*-benzoquinone (DDQ) or $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, but failed to convert **5** to **6** under these conditions.¹⁷ In this study, we attempted to identify whether **3** can be converted to **1** and **2** under enzymatic or chemical dimerization.¹⁶ When compound **3** was incubated with lysates of a dinapinone-producing fungus, **1** and **2** were found to be produced time-dependently in a ratio of 2:3 (Figure 7a); thus, the absolute stereochemistry of **1** and **2** was proved

to be *M*,3*S*,13*R*,15*R* and *P*,3*S*,13*R*,15*R*, respectively. In contrast, under chemical dimerization conditions using $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, **3** was not converted to **1** and **2**. These results indicated that atropisomers **1** and **2** were enzymatically produced from **3**.

METHODS

General

Various NMR spectra were obtained using an Agilent Technologies XL-400 (400 MHz) spectrometer (Agilent Technologies, Santa Clara, CA, USA). FAB-MS was conducted on a JEOL JMS-700 Mstation spectrometer (JEOL, Tokyo, Japan). Electrospray ionization mass spectrometry was conducted on a JEOL JMS-T100LP spectrometer. UV-visible and IR spectra were measured with a Beckman DU640 spectrophotometer (Beckman Coulter, Inc., Fullerton, CA, USA) and a Horiba FT-210 Fourier transform infrared spectrometer (Horiba, Kyoto, Japan), respectively. Optical rotations and circular dichroism were recorded on a JASCO model DIP-181 polarimeter (JASCO, Tokyo, Japan) and J-720 circular dichroism spectrometer (JASCO).

Microorganism

The dinapinone- and monapinone-producing fungus *P. pinophilum* FKI-3864 was isolated from a soil sample collected in Hilo, Hawaii, USA.⁶

Fermentation of dinapinone A1 (**1**) and A2 (**2**) and monapinone A (**3**) and B (**4**)

The culture conditions for the production of **1** to **4** were described previously.^{7,8} Strain FKI-3864 was fermented in media containing seawater or tap water. Dimers **1** and **2** (12 mg l^{-1}) were produced for 10–20 days in tap water-containing medium. On the other hand, monomers **3** and **4** (337 and 8 mg l^{-1}) were produced for 20–30 days in seawater-containing medium.

Isolation of dinapinone A1 (**1**) and A2 (**2**)

The purification procedures of **1** and **2** were described previously.⁷ Bioassay-guided fractionation of the culture broth of the fungus, including organic solvent extraction, silica gel column chromatography, ODS gel column chromatography and reversed phase (C18 and C30) HPLC, yielded **1** and **2**.

Dinapinone A1 (**1**). Yellow powder; $[\alpha]_{\text{D}}^{26} -190.1^\circ$ (c 0.1, MeOH); IR (KBr) ν_{max} 3400, 2925, 1637 cm^{-1} ; UV (MeOH) λ_{max} 223 (ϵ 27,300), 269 (ϵ 93,000), 382 (ϵ 27,300); ^1H and ^{13}C NMR data (Tables 1 and 2); CD (CHCl_3) λ_{ex} termum ($\Delta\epsilon$) 280 (-70.9), 251 ($+84.7$); FAB-MS m/z 835 ($\text{M} + \text{H}^+$); HRFAB-MS m/z for $\text{C}_{46}\text{H}_{59}\text{O}_{14}$ ($\text{M} + \text{H}^+$), calculated: 835.3905, found: 835.3923.

Dinapinone A2 (**2**). Yellow powder; $[\alpha]_{\text{D}}^{26} +243.6^\circ$ (c 0.1, MeOH); IR (KBr) ν_{max} 3398, 2924, 1637 cm^{-1} ; UV (MeOH) λ_{max} 223 (ϵ 24,800), 269 (ϵ 80,000), 382 (ϵ 22,300); CD (CHCl_3) λ_{ex} termum ($\Delta\epsilon$) 278 ($+47.6$), 255 (-69.2); ^1H and ^{13}C NMR data (Tables 1 and 2); FAB-MS m/z 835 ($\text{M} + \text{H}^+$); HRFAB-MS m/z for $\text{C}_{46}\text{H}_{59}\text{O}_{14}$ ($\text{M} + \text{H}^+$), calculated: 835.3905, found: 835.3934.

Isolation of monapinone A (**3**) and B (**4**)

The purification procedures of **3** and **4** were described previously.⁸ LCUV analysis-guided fractionation of the culture broth of the fungus, including organic solvent extraction, ODS gel column chromatography and reversed phase (C18) HPLC, yielded **3** and **4**.

Monapinone A (**3**). Light brown powder; $[\alpha]_{\text{D}}^{26} +1.64^\circ$ (c 0.10, CHCl_3); IR (KBr) ν_{max} 3435, 2927, 1633 cm^{-1} ; UV (MeOH) λ_{max} 220 (ϵ 62,600), 262 (ϵ 162,600), 370 (ϵ 42,700); CD (CHCl_3) λ_{ex} termum ($\Delta\epsilon$) 217 (-5.6), 238 ($+2.1$), 266 (-6.0), 342 ($+6.0$); ^1H and ^{13}C NMR data (Tables 1 and 2); ESI-TOF-MS m/z 417 ($\text{M} - \text{H}^-$); HRESI-TOF-MS m/z for $\text{C}_{23}\text{H}_{29}\text{O}_7$ ($\text{M} - \text{H}^-$), calculated: 417.1923, found: 417.1925.

Monapinone B (**4**). Light brown powder; $[\alpha]_{\text{D}}^{26} +1.80^\circ$ (c 0.10, CHCl_3); IR (KBr) ν_{max} 3435, 2929, 1639 cm^{-1} ; UV (MeOH) λ_{max} 222 (ϵ 27,700), 261 (ϵ 78,500), 374 (ϵ 18,600); CD (CHCl_3) λ_{ex} termum ($\Delta\epsilon$) 217 (-5.6), 238 ($+1.9$), 266 (-6.0), 342 ($+6.0$); ^1H and ^{13}C NMR data (Tables 1 and 2); ESI-TOF-MS m/z 373 ($\text{M} - \text{H}^-$); HRESI-TOF-MS m/z for $\text{C}_{21}\text{H}_{25}\text{O}_6$ ($\text{M} - \text{H}^-$); calculated: 373.1651, found: 373.1650.

Preparation of acetonide derivative of **1**

To a solution of **1** (6.5 mg, 0.0078 mmol) in DMF (240 μl) was added acetone dimethyl acetyl (80 μl , 0.1 M) and PPTS (0.2 mg, 0.00078 mmol) at room temperature. The mixture was stirred at room temperature for 24 h, and then extracted with ethyl acetate. The ethyl acetate extract was evaporated to give an acetonide derivative of **1** (6.6 mg, 92.7%); HRESI-TOF-MS m/z for $\text{C}_{52}\text{H}_{66}\text{O}_{14}$ ($\text{M} - \text{H}^-$), calculated: 913.4374, found: 913.4349.

Lysate preparation of *P. pinophilum* FKI-3864

The 28-day-old whole culture broth of strain FKI-3864 was centrifuged at 3,000 r.p.m. to obtain the mycelium, which was suspended in four volumes of 100 mM sodium citrate buffer (pH 5.0) and disrupted by sonication using Bioruptor (Cosmo Bio Co. Ltd, Tokyo, Japan) with four 150-s bursts with intermittent cooling for 5 min on ice. The resulting suspension was centrifuged at 12,000 r.p.m. for 30 min at 4°C , and the supernatant (lysate) was used as an enzyme source for *in vitro* dimerization reaction of **3**.

In vitro dimerization conditions

The reaction mixture containing 100 mM sodium citrate buffer (pH 5.0), compound **3** (5 μg) and mycelial lysate (70 μg preprotein) or 125 μM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in a total volume of 250 μl was incubated at 27°C with agitation at 120 r.p.m. The reaction was stopped at the indicated times by adding ethyl acetate (500 μl) and water (250 μl), and the solutions were mixed well. The organic layer (400 μl) containing the products was evaporated, and the residue was dissolved in methanol (100 μl) to analyze the products by HPLC, as described below.

Analytical conditions by HPLC

HPLC system (ELITE LaChrom; Hitachi High-Technologies Co., Tokyo, Japan), column, Develosil C30 (i.d. $4.6 \times 250 \text{ mm}$; Nomura Chemical Co., Aichi, Japan); mobile phase, 80% $\text{CH}_3\text{CN}/0.05\% \text{ H}_3\text{PO}_4$; flow rate, 1 ml min $^{-1}$; detection, UV at 270 nm; sample volume, 5 μl . Compounds **1**, **2** and **3** were eluted as peaks with retention times of 15.2, 19.5 and 12.0 min, respectively.

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