ORIGINAL ARTICLE

Wakodecalines A and B, new decaline metabolites isolated from a fungus *Pyrenochaetopsis* sp. RK10-F058

Toshihiko Nogawa[1], Naoki Kato[2], Takeshi Shimizu[1], Akiko Okano[1], Yushi Futamura[1], Shunji Takahashi[2] and Hiroyuki Osada[1]

Two new decaline metabolites, wakodecalines A and B, were isolated from a fungus, *Pyrenochaetopsis* sp. RK10-F058, by screening for structurally unique metabolites using LC/MS analysis. Their structures were determined on the basis of NMR and mass spectrometric measurements. The absolute structures were confirmed by a combination of chemical methods including chemical degradation, a modified Mosher's method and Marfey's method, and comparison of the experimental electronic CD (ECD) spectrum with calculated one. Both compounds had a cyclopentanone-fused decaline skeleton and an *N*-methylated amino acid moiety derived from a serine. They showed moderate antimalarial activity against the *Plasmodium falciparum* 3D7 strain.
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INTRODUCTION

Microorganisms produce structurally unique metabolites with various biological activities.[1–3] These metabolites have been used as agrochemicals, pharmaceutical leads and therapeutic agents.[4] They are also important as bioprobes, the tools used to investigate biological functions in chemical biology studies.[5] Numerous secondary metabolites have been isolated using various bioassay-guided separations. We have isolated new active metabolites such as reveromycins,[6] epoxyquinols[7] and azaspirene[8] from actinomycetes and fungi using unique bioassay systems. However, it is difficult to discover active metabolites as they are less abundantly found, form complex mixtures in extract or are unsuitable for bioassay systems. To overcome this difficulty, we constructed a microbial metabolite fraction library comprising semipurified metabolites coupled with an in-house spectral database, Natural Products Plot (NPPlot),[9,10] that contains UV and mass spectral information on metabolites of each microbe for structure-oriented isolation. Using the NPPlot, we isolated various structurally unique metabolites, such as verticilactam,[11] spiroamides[12] and pyrrolizilactone.[13] In addition, we expanded the library by stocking broths prepared from microbes isolated from soils from various regions of Japan. Approximately 6000 extracts of the broth library were analyzed using diode-array detector LC/MS and subsequently screened for structurally interesting metabolites. During screening, a broth prepared from a fungus, *Pyrenochaetopsis* sp. RK10-F058, was found to produce interesting metabolites, one of which was possibly identical to phomasetin[14] by UV and mass spectral data. The others were likely to be related to phomasetin with *m/z* of 400–500.

Phomasetin is a bioactive metabolite isolated from the fungus, *Phoma* sp.; it comprises a tetramic acid and a decaline unit.[14] We recently identified Diels–Alderase as a key enzyme that controls the stereoselective construction of the decaline skeleton of equisetin[15] that had a similar skeleton to that of phomasetin.[16] Interestingly, phomasetin has a completely opposite absolute structural configuration to that of equisetin, and this is interesting from the viewpoint of biosynthesis. Therefore, we focused on the decaline-related metabolites produced by *Pyrenochaetopsis* sp. RK10-F058, and isolated new metabolites designated as wakodecalines A (**1**) and B (**2**) along with phomasetin (**3**) (Figure 1). Here, we report the isolation and structures of compounds **1** and **2**.

RESULTS AND DISCUSSION

The fungal strain *Pyrenochaetopsis* sp. RK10-F058 collected in Wako, Saitama, Japan, was cultured in 4.8 l of culture medium for 13 days. The culture broth was partitioned thrice with half the volume of EtOAc and the resulting organic extract was evaporated *in vacuo* to obtain a light brown oil (1.30 g). The oil was separated into 8 fractions using SiO₂ medium pressure liquid chromatography (MPLC) with a CHCl₃/MeOH stepwise gradient. The fourth and fifth fractions were combined and subjected to C18-MPLC with an acetonitrile/0.05% aqueous formic acid gradient system to obtain seven subfractions. The fourth subfraction was separated using Sephadex LH-20 (GE Healthcare, Pittsburgh, PA, USA) to obtain four fractions. The third and second fractions were purified using C18-HPLC with acetonitrile/0.05% formic acid isocratic elution at 72:28 and 48:52 to obtain

[1]RIKEN Center for Sustainable Resource Science, Chemical Biology Research Group, Wako, Saitama, Japan and [2]RIKEN Center for Sustainable Resource Science, Natural Product Biosynthesis Research Unit, Saitama, Japan
Correspondence: Dr H Osada, RIKEN Center for Sustainable Resource Science, Chemical Biology Research Group, 2-1, Hirosawa, Saitama 351-0198, Japan.
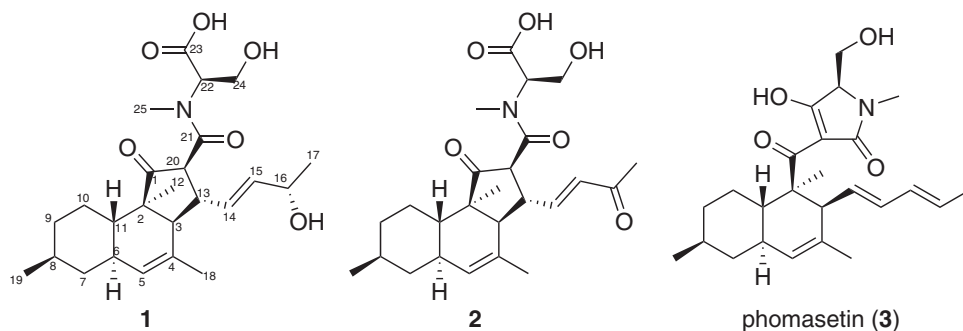E-mail: hisyo@riken.jp

Figure 1 Structures of wakodecalines A (**1**) and B (**2**) and phomasetin (**3**).

Table 1 Physicochemical properties of wakodecalines A (1) and B (2)

	1	2
Appearance	Colorless powder	Colorless powder
Optical rotation (MeOH)	$[\alpha]_{589}^{27}$−31.8 (*c* 0.1)	$[\alpha]_{589}^{26}$+1.4 (*c* 0.1)
Molecular formula	$C_{25}H_{37}NO_6$	$C_{25}H_{35}NO_6$
UV (MeOH) λ_{max} (log ε) (nm)	end	237sh (4.84)
ECD (MeOH) λ_{max} ($\Delta\varepsilon$) (nm)	296 (−2.68), 340 (0)	234 (+10.9), 296 (−2.98), 340 (0)
IR (ATR) ν_{max} (cm^{-1})	3400–3500, 2945, 2920, 2855, 1730, 1625, 1455, 1405, 1375, 1200, 1120	3400–3500, 2945, 2920, 2845, 1730, 1625, 1450, 1405, 1365, 1250
ESIMS (*m/z*)	448 [M+H]$^+$	446 [M+H]$^+$
HRESITOFMS (*m/z*)	Found 446.2545 [M-H]$^-$	Found 444.2392 [M-H]$^-$
	Calcd $C_{25}H_{36}NO_6$, 446.2543	Calcd $C_{25}H_{34}NO_6$, 444.2386

Abbreviations: ECD, electronic CD; ESIMS, electrospray ionization MS; HRESITOFMS, high-resolution electrospray ionization time-of-flight MS.

compounds **1** (77.7 mg) and **2** (26.8 mg), respectively, as colorless powders (Table 1 and Supplementary Figure S1). Compound **3** was isolated as a colorless powder (397.3 mg) from the first fraction of the SiO₂ MPLC fractions using SiO₂ MPLC (hexane/EtOAc) and Sephadex LH-20 separations and identified as phomasetin using the NMR and electronic CD (ECD) spectra.[14]

The molecular formula of **1** was determined to be $C_{25}H_{37}NO_6$ using high-resolution electrospray ionization time-of-flight MS (HRESI-TOFMS) (found *m/z*: 446.2545 [M–H]$^-$, calcd for $C_{25}H_{36}NO_6$ 446.2543) (Supplementary Figure S2). The IR spectrum implied the presence of hydroxyl and carbonyl groups (3400–3500, 1730 and 1625 cm^{-1}). The ^{1}H and ^{13}C NMR spectra of **1** in acetonitrile-d_3 showed doubling and broadening signals (Supplementary Figures S3 and S4). These features were reported for those of phomasetin,[14] equisetin[16] and gabusectin[17] that had a tetramic acid and a decaline unit in the molecules, suggesting that **1** was supposed to have a related substructure to phomasetin. The ^{1}H NMR spectrum showed 5 methyl signals at δ 0.88 (d, *J* = 6.3 Hz), 0.90 (s), 1.14 (d, *J* = 6.3 Hz), 1.71 (s) and 3.09 (s) that was assigned as an *N*-methyl, and 3 olefin signals at δ 5.22 (brs), 5.53 (dd, *J* = 15.5, 6.3 Hz) and 5.69 (dd, *J* = 15.5, 9.2 Hz). It also contained an α-proton at δ 4.81 (dd, *J* = 8.0, 5.1 Hz), indicating the presence of an amino acid. Most signals in the ^{13}C NMR spectrum were observed as doublings with a signal strength of ∼2:1. The careful assignment of heteronuclear single quantum correlation (HSQC) spectrum through the ^{13}C DEPT experiment led to the assignment of the 25 carbon signals as the main conformers (Supplementary Figures S5 and S6). It comprised 5 methyls, 4 methylenes including an oxygenated one at δ 61.0, 11 methines including an oxygenated one at δ 68.9, suggesting the presence of a hydroxyl group, and 5 quaternary

signals that included three carbonyl signals at δ 171.4, 171.7 and 215.4. The carbon signals also contained 4 olefin signals at δ 128.3, 132.6, 134.3 and 138.2, and an α-carbon signal at δ 61.4 of an amino acid. These observations implied that **1** had a decaline skeleton and an *N*-methylated serine similar to that of **3**. The detailed structure was confirmed by interpreting the double quantum filtered (DQF) COSY and heteronuclear multiple bond correlation (HMBC) spectra (Supplementary Figures S7 and S8). The DQF-COSY revealed the proton spin networks of a cyclohexane moiety from C-5 to C-11 including Me-19 attached at C-8, an olefin side chain from C-13, which also connected to C-3 and C-20, to C-17, and a primary alcohol at C-22 (Figure 2a). The decaline ring was confirmed by HMBC correlations from H-3 to C-2 and C-11, from H-5 to C-3 and from Me-18 to C-3, C-4 and C-5 (Figure 2a). Me-12 showed HMBC correlations to C-2, C-3 and C-11, suggesting that Me-12 was attached at the C-2. Me-12 also showed HMBC correlation to C-1 that was correlated from H-20, and these correlations led to the construction of a cyclopentanone-fused decaline skeleton. The *N*-methylated serine moiety was confirmed by HMBC correlations from H-22 and H-24 to C-23, from H-22 to C-21 and from *N*-Me-25 to C-21 and C-22 and considering the ^{13}C NMR chemical shifts. It was confirmed to attach to C-20 by HMBC correlation from H-20 to C-21 and the planer structure was constructed.

The geometry at Δ^{14} was assigned as an *E*-configuration by the large coupling constant of 15.5 Hz and confirmed by NOESY correlation between H-13 and H-15. The relative stereochemistry of the decaline moiety was determined using the NOESY spectrum (Supplementary Figure S9). The decaline skeleton was confirmed as a *trans*-configuration by NOESY correlations between the H-6 and H-8, H-10ax and
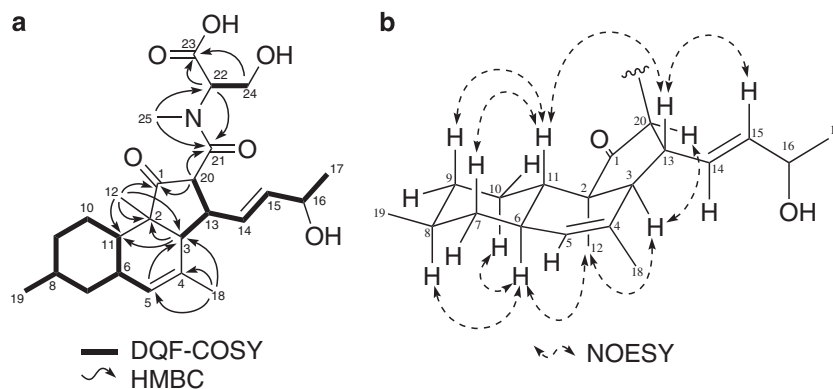
Figure 2 Key DQF-COSY and HMBC correlations (**a**) and NOESY correlations (**b**).

Me-12 and between H-11 and H-7ax and H-9ax (Figure 2b). These correlations also confirmed a chair form of the cyclohexane ring, α-configuration of Me-12 and β-configuration of Me-19. The cyclopentanone ring fused by a β-configuration to the decaline was assigned by NOESY correlations between the H-3 and Me-12 and between H-11 and H-13 that also confirmed an α-configuration of the olefin side chain at C-13. The N-methylated serine moiety was assigned as a β-configuration at C-20 by a NOESY correlation between H-3 and H-20. Therefore, the overall structure of **1**, including the relative stereochemistry around the decaline moiety, was determined as ($2R^*$, $3S^*$, $6R^*$, $8S^*$, $11S^*$, $13R^*$ and $20S^*$).

The molecular formula of **2** was determined to be $C_{25}H_{35}NO_6$ by HRESITOFMS (found m/z: 444.2392 [M-H]$^-$, calcd for $C_{25}H_{34}NO_6$ 444.2386), which was lower than that of **1** by two hydrogen (Supplementary Figure S10). The ^1H and ^{13}C NMR spectra were almost identical to those of **1** showing doubling and broadening signals except for the signals assigned for the olefin side chain at C-13 (Supplementary Figures S11 and S12). The proton and carbon signals derived from the hydroxyl group at C-16 of **1** disappeared and the additional carbonyl signal was observed at δ 199.0. These observations suggested that **2** had a carbonyl group at C-16 that was oxidized from the hydroxyl group of **1**. The planer structure was confirmed in the same manner as that of **1** based on the interpretation of NMR spectra including ^{13}C DEPT, HSQC, DQF-COSY and HMBC (Supplementary Figures S13–S16). The olefin side chain was confirmed by the HMBC correlations from Me-17 to C-15 and C-16 (Supplementary Figure S17a). The relative stereochemistry, including the geometry of Δ^{14}, was confirmed to be the same as that of **1** by the NOESY correlations (Supplementary Figures S17b and S18).

The absolute configurations of **1** and **2** were confirmed using a combination of chemical methods and the calculation of the ECD spectrum. The configuration at C-16 of **1** was determined by application of the modified Mosher's method.[18] The methyl ester **4** derived from **1** by treatment with $TMSCHN_2$ was converted to the (R, R) and (S, S)-diMTPA esters (**5** and **6**) at C-16 and C-24 with (S)- and (R)-MTPA chloride, respectively (Scheme 1). They were used to calculate the differences of ^1H NMR chemical shift values around C-16. The results suggested that the absolute configuration at C-16 was assigned as an S (Figure 3). The Marfey's method[19] was applied to determine the absolute configuration at C-22 on the N-methylated serine derived from **1** by hydrolysis using 6 N HCl for 18 h at 110 °C in a sealed ampule. The hydrolytic product, N-methylated serine, was reacted with 1-fluoro-2,4-dinitrophenyl-5-L-leucinamide (L-FDLA), and the resulting product, N-methylated serine-L-FDLA, was analyzed using LC/MS. At the same time, the standard L-FDLA derivatives of

N-methylated L- and D-serines, which were derived from N-carbobenzoxy (cbz) L- and D-serines, were prepared and analyzed using LC/MS. The FDLA derivative of N-methylated serine from **1** eluted at 16.20 min, whereas the standard L- and D-derivatives eluted at 15.76 and 16.19 min, respectively (Supplementary Figure S19). The result showed that the N-methylated serine in **1** was the D-form and C-22 was assigned as an R-configuration. Compound **2** was confirmed to have the same absolute configurations as those of **1**, except for the C-16, by direct comparison of the methyl ester **7**, which was derived from **2** by treatment with $TMSCHN_2$, with an oxidized derivative of the methyl ester **4** using the ^1H NMR and ECD spectra (Scheme 1 and Supplementary Figure S20).

The absolute configuration of the decaline skeleton was deduced by comparing the experimental and calculated ECD spectra of **2**.[20] The conformers obtained by conformational analysis using Merck molecular force field, which gave 123 stable conformers, were optimized by Hartree–Fock STO-3G and further optimized by density functional theory at B3LYP/6-31G(d) level. The ECD spectra were simulated by time-dependent density functional theory at ωB97XD/6-31G(d) level and Boltzmann averaged. The resulting calculated ECD spectrum agreed with the experimental ECD spectrum (Figure 4). Based on the above results, the absolute configurations were determined to be 2R, 3S, 6R, 8S, 11S, 13R, 16S, 20S, 22R for **1** and 2R, 3S, 6R, 8S, 11S, 13R, 20S, 22R for **2**, respectively, and found to be the same as those of phomasetin on the decaline moiety and N-methylated serine.

We evaluated the cytotoxicities of compounds **1** and **2** as well as those of **3** against the human cervical epidermoid carcinoma cell line, HeLa, human promyelocytic leukemia cell line, HL-60, and rat kidney cells infected with ts25, a T-class mutant of Rous sarcoma virus Prague strain, src^{ts}-NRK. Furthermore, their antimicrobial activities against Staphylococcus aureus 209, Escherichia coli HO141, Aspergillus fumigatus Af293, Pyricularia oryzae kita-1 and Candida albicans JCM1542, and antimalarial activity against P. falciparum 3D7 were evaluated. Compounds **1** and **2** did not show cytotoxicity or antimicrobial activity with IC_{50} of 30 μg ml^{-1}, but they showed moderate antimalarial activity with IC_{50} values of 28 and 16 μg ml^{-1}, respectively. Compound **3** showed moderate cytotoxicity against HeLa, HL-60 and src^{ts}-NRK with IC_{50} values of 4.5, 1.4 and 4.4 μg ml^{-1}, strong antimicrobial activities against S. aureus, E. coli and P. oryzae with IC_{50} values of 0.057, 0.53 and 0.018 μg ml^{-1} and strong antimalarial activity with an IC_{50} value of 0.74 μg ml^{-1}. These results suggest that the tetramic acid unit might be important for the observed activities.

The broadening and doubling of signals in ^1H and ^{13}C NMR spectra of wakodecalines A and B suggested the existence of conformers. These features are reported for phomasetin[14] and other

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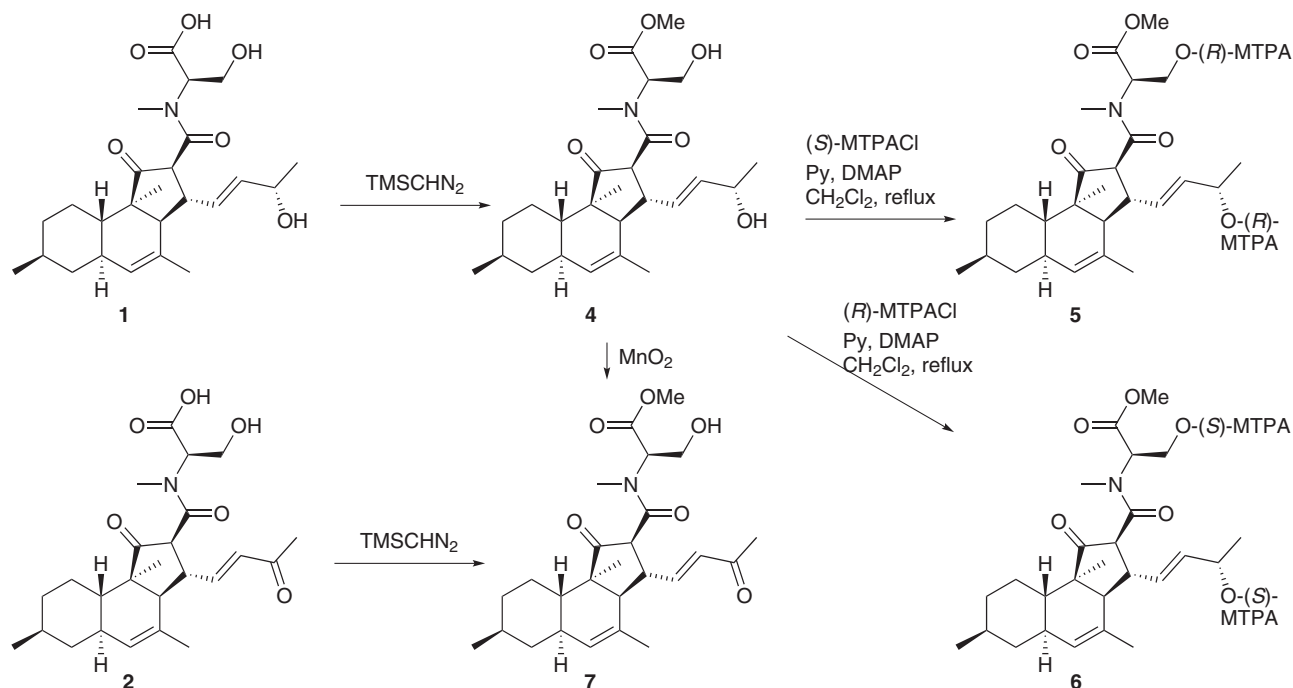
Scheme 1 Preparation of 16,24-di(R)-MTPA ester **5**, 16,24-di(S)-MTPA ester **6** and methyl ester **7**.
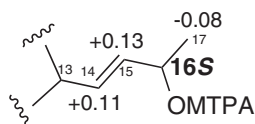

Figure 3 $\Delta\delta_{S-R}$ values obtained from 16,24-diMTPA esters **5** and **6**.
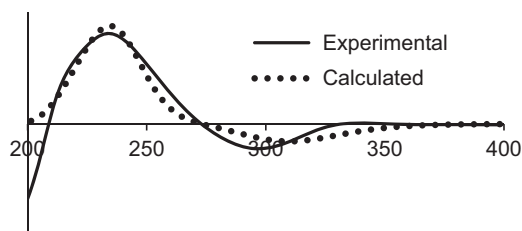

Figure 4 Experimental and calculated electronic CD (ECD) spectra of wakodecaline B (**2**).

tetramic acid containing decaline metabolites as mentioned before, and known to be caused by tautomerization.[16,17] Tetramic acid is reported to be a mixture of tautomers of a diketo form and enolized hydroxyl forms in the solution.[21] Similar tautomerizations have been suggested for a ketone at C-1 position of phomasetin and equisetin.[14,16] From these reports, the diketo moiety of wakodecalines A and B is possibly associated with enolized hydroxyl-forms at C-1 or C-21.

Wakodecalines A and B have a cyclopentanone fused decaline skeleton that is rare in natural products. Only two fungal metabolites, fusarisetin A[22,23] and altercrasin A,[24] have been reported as natural products with the same skeleton. However, the absolute stereochemistry of their decaline moiety was identical to that of equisetin, but opposite to those of the wakodecalines. D-Serine was assigned as the amino acid that is also the opposite of equisetin. From the above-mentioned facts, we proposed that the wakodecalines might be biosynthesized from phomasetin, which has the same absolute stereochemistry as that of the wakodecalines, through a spiro

intermediate similar to altercrasin A[24] (Supplementary Scheme S1). We will continue to search related metabolites from this fungus to reveal the biosynthetic mechanism of decaline-type tetramic acid derivatives and examine their activities.

EXPERIMENTAL PROCEDURES

General experimental procedures

Analytical-grade solvent and reagents were purchased from commercial sources. UV and optical rotations were recorded on a JASCO V-630 BIO spectrophotometer (JASCO International, Tokyo, Japan) and a HORIBA SEPA-300 high sensitive polarimeter (HORIBA, Kyoto, Japan), respectively. ECD spectra were measured on a JASCO J-720 CD spectrometer. IR spectra were recorded on a HORIBA FT-720 with a DuraSampl IR II ATR instrument. NMR data were obtained at 500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR on a JEOL JNM-ECA-500 spectrometer (JEOL, Tokyo, Japan). Chemical shifts (in ppm) were referenced against the residual undeuterated solvent. LC/MS analysis was performed on a Waters 2965 Alliance system with 2996PDA detector (Waters, Milford, MA, USA) connected to AB Sciex Qtrap by ESI probe (AB Sciex, Framingham, MA, USA) on a Waters Xterra C18 column (2.1 mm i.d. × 150 mm, 5 μm) with elution of acetonitrile/0.05% aqueous formic acid linear gradient system (acetonitrile: 5 to 100% in 30 min at 0.2 ml min^{-1}), or Waters 600E pump system with 996 PDA detector connected to Waters ZQ by ESI probe on a Senshu Pak Pegasil ODS column (Senshu Scientific, Tokyo, Japan) (4.6 mm i.d. × 250 mm, 5 μm) with elution of acetonitrile/0.05% aqueous formic acid linear gradient system (acetonitrile: 20 to 100% in 20 min at 1.0 ml min^{-1}). HRESITOFMS was obtained on a Waters Synapt G2. Teredyne ISCO CombiFlash Companion (Teredyne ISCO, Lincoln, NE, USA) was used for MPLC. Preparative HPLC was performed using a Waters 600E pump system with Senshu Pak Pegasil ODS column (20 mm i.d. × 250 mm or 10 mm i.d. × 250 mm, 5 μm).

Strain and culture condition

The fungal strain *Pyrenochaetopsis* sp. RK10-F058 was isolated from a soil sample collected in Wako, Saitama, Japan, in 2010, and identified using ITS-5.8s rDNA sequencing and morphological observation (Techno Suruga Laboratory, Shizuoka, Japan). It was deposited in Chemical Biology Research Group, RIKEN. The conidia freshly prepared was inoculated in 500 ml

cylindrical flasks containing 100 ml of PDB medium with 0.1% agar at 28 °C for 6 days on a rotary shaker at 150 r.p.m. Then, 4 ml of the preculture was inoculated in 500 ml cylindrical flasks containing 100 ml of YMGS medium (0.5% yeast extract, 0.5% malt extract, 1% glucose and 1% soluble starch), and cultured at 28 °C for 13 days on the same rotary shaker.

Extraction and isolation

The 4.8 l of culture broth was partitioned with half volume of EtOAc three times to obtain an organic soluble material. It was evaporated *in vacuo* to afford 1.30 g of light brown oil. The brown oil was subjected to SiO_2 MPLC with $CHCl_3$/MeOH stepwise gradient system (MeOH: 0, 1, 2, 5, 10, 20, 50 and 100%) to afford 8 fractions. The first fraction was separated by SiO_2 MPLC with hexane/EtOAc linear gradient and Sephadex LH-20 to afford 397.3 mg of compound **3** as colorless powder. The fourth and fifth fractions was combined and subjected to C18-MPLC with acetonitrile/0.05% aqueous formic acid linear gradient to obtain seven subfractions. The fourth subfraction was separated by Sephadex LH-20 to afford four fractions. The third and second fractions were purified by C18-HPLC with acetonitrile/0.05% aqueous formic acid isocratic elution of 72:28 and 48:52 to afford 77.7 and 26.8 mg of compounds **1** and **2** as colorless powder, respectively. Physicochemical properties of **1** and **2** are summarized in Table 1. ^{1}H and ^{13}C NMR chemical shifts in acetonitrile-d_3 of **1** and **2** are summarized in Table 2.

Table 2 ^{1}H and ^{13}C NMR chemical shifts of wakodecalines A (**1**) and B (**2**) in acetonitrile-d_3

Position	δ_C (1)	δ_H (multi, *J* in Hz) (1)	δ_C (2)	δ_H (multi, *J* in Hz) (2)
1	215.4	—	213.9	—
2	53.3	—	53.0	—
3	55.2	2.11 (d, 10.9)	54.5	2.26 (brd, 10.8)
4	134.3	—	133.6	—
5	128.3	5.22 (brs)	128.6	5.25 (brs)
6	38.7	1.83 (m)	38.3	1.84 (m)
7	43.3	0.76 ax. (ddd, 12.0, 12.0, 12.0)	42.9	0.77 ax. (m)
		1.79 eq.		1.79 eq. (m)
8	34.0	1.43 (m)	33.7	1.43 (m)
9	36.6	0.82 ax. (m)	36.3	0.81 ax. (m)
		1.72 eq. (m)		1.72 eq. (m)
10	26.3	1.04 ax. (dddd, 12.6, 12.6, 12.6, 3.4)	25.9	1.07 ax. (m)
		1.40 eq. (m)		1.40 eq. (m)
11	39.2	1.42 (m)	39.0	1.40 (m)
12	15.8	0.90 (3H, s)	15.4	0.93 (3H, s)
13	51.2	3.06 (m)	49.9	3.32 (ddd, 10.3, 10.3, 10.3)
14	132.6	5.69 (dd, 15.5, 9.2)	149.6	6.91 (dd, 16.0, 9.7)
15	138.2	5.53 (dd, 15.5, 6.3)	133.0	6.11 (d, 16.0)
16	68.9	4.17 (m)	199.0	—
17	23.9	1.14 (3H, d, 6.3)	27.7	2.20 (3H, s)
18	24.7	1.71 (3H, s)	24.0	1.68 (3H, s)
19	23.1	0.88 (3H, d, 6.3)	22.7	0.88 (3H, d, 6.9)
20	59.9	3.68 (d, 10.3)	58.7	3.82 (m)
21	171.4	—	170.0	—
22	61.4	4.81 (dd, 8.0, 5.1)	61.0	4.83 (dd, 6.9, 5.2)
23	171.7	—	171.2	—
24	61.0	3.88 (2H, m)	60.6	3.82 (m)
				3.87 (m)
25	35.6	3.09 (3H, s)	34.9	3.08 (3H, s)

3: colorless powder; $[\alpha]^{22}_{589}$ (*c* 0.1, MeOH) +558; ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 234 (+18.2), 260 (+6.46), 293 (+15.1), 360 (0); ^{1}H NMR ($CDCl_3$) δ 0.87 (m), 0.89 (d, *J* = 6.3 Hz, 3H), 1.01 (m), 1.08 (m), 1.36 (m), 1.49 (m), 1.63 (m), 1.66 (d, *J* = 6.3 Hz, 3H), 1.72 (m), 1.77 (m), 1.80 (m), 1.94 (m), 3.01 (s, 3H), 3.12 (brd, *J* = 8.6 Hz), 3.60 (brs), 3.86 (m), 3.99 (m), 5.16 (m), 5.21 (m), 5.48 (m), 5.77 (m), 5.87 (m); ^{13}C NMR ($CDCl_3$) δ 14.1, 18.2, 22.4, 22.7, 27.5, 28.4, 33.7, 35.9, 39.4, 39.8, 42.7, 49.4, 49.6, 60.6, 66.9, 100.1, 126.3, 128.1, 130.4, 131.6, 131.7, 132.6, 177.3, 190.8, 199.2.

Preparation of methyl ester 4

To a stirred solution of **1** (4.8 mg, 10.7 μM) in benzene-MeOH (0.6 ml, 4:1) was added 2.0 M solution of $TMSCHN_2$ in diethyl ether (10.8 μl; 21.6 μmol) at room temperature. The reaction mixture was stirred for 10 min and then concentrated to provide methyl ester **4** (4.9 mg, 99%).

4: ^{1}H NMR ($CDCl_3$) δ 0.87 (d, *J* = 6.5 Hz, 3H), 0.95 (s, 3H), 1.25 (d, *J* = 6.5 Hz, 3H), 1.71 (brs, 3H), 2.01 (d, *J* = 11.0 Hz, 1H), 3.18 (s, 3H), 3.28 (m, 1H), 3.48 (d, *J* = 10.5 Hz, 1H), 3.71 (s, 3H), 3.96 (m, 1H), 4.06 (m, 1H), 4.27 (m, 1H), 4.96 (dd, *J* = 7.5, 5.5 Hz, 1H), 5.21 (brs, 1H), 5.65 (dd, *J* = 15.0, 5.0 Hz, 1H), 5.67 (dd, *J* = 15.0, 7.5 Hz, 1H).

Preparation of 16,24-di(*R*)-MTPA ester 5

To a stirred solution of crude **4** (1.6 mg, 3.5 μmol) in CH_2Cl_2 (0.5 ml) were added pyridine (1.8 μl, 20.8 μmol), DMAP (0.1 mg) and (*S*)-MTPA chloride (2.6 μl, 13.8 μmol) at room temperature. The reaction mixture was heated under reflux for 12 h. The reaction mixture was evaporated to afford a yellow oil that was purified by preparative TLC (*n*-hexane: EtOAc = 2:1) to provide 16,24-di(*R*)-MTPA ester **5** (0.3 mg, 10%).

5: ^{1}H NMR ($CDCl_3$) δ 0.88 (d, *J* = 6.5 Hz, 3H), 0.93 (s, 3H), 1.39 (d, *J* = 6.5 Hz, 3H), 1.64 (brs, 3H), 2.94 (s, 3H), 3.30 (m, 2H), 3.51 (s, 3H), 3.53 (s, 3H), 3.57 (s, 3H), 4.61 (dd, *J* = 12.0, 3.0 Hz, 1H), 4.81 (dd, *J* = 12.0, 6.0 Hz, 1H), 4.98 (dd, *J* = 6.0, 3.0 Hz, 1H), 5.19 (brs, 1H), 5.56 (m, 1H), 5.57 (dd, *J* = 15.5, 6.5 Hz, 1H), 5.63 (dd, *J* = 15.5, 5.5 Hz, 1H).

Preparation of 16,24-di(*S*)-MTPA ester 6

This compound was prepared from **4** with (*R*)-MTPA chloride in 10% yield using the same procedure for preparation of **5**.

6: ^{1}H NMR ($CDCl_3$) δ 0.88 (d, *J* = 6.5 Hz, 3H), 0.92 (s, 3H), 1.31 (d, *J* = 7.0 Hz, 3H), 1.67 (brs, 3H), 3.00 (s, 3H), 3.37 (m, 2H), 3.50 (s, 6H), 3.60 (s, 3H), 4.67 (dd, *J* = 12.5, 4.0 Hz, 1H), 4.75 (dd, *J* = 12.5, 6.0 Hz, 1H), 5.09 (m, 1H), 5.21 (brs, 1H), 5.55 (m, 1H), 5.70 (dd, *J* = 15.0, 5.0 Hz, 1H), 5.74 (dd, *J* = 15.0, 7.0 Hz, 1H).

Oxidation of methyl ester 4

To a stirred solution of methyl ester **4** (0.8 mg, 1.7 μM) in CH_2Cl_2 (0.2 ml) was added active MnO_2 (1.2 mg, 13.8 μmol) at room temperature. After stirring for 1 h, the mixture was purified by SiO_2 column chromatography (*n*-hexane: EtOAc = 1:3) to provide colorless solid **7** (0.6 mg, 75%).

7: ^{1}H NMR ($CDCl_3$) δ 0.88 (d, *J* = 6.5 Hz, 3H), 0.98 (s, 3H), 1.69 (brs, 3H), 2.12 (d, *J* = 9.5 Hz, 1H), 2.25 (s, 3H), 2.48 (brt, *J* = 3.0 Hz, 1H), 3.18 (s, 3H), 3.54 (m, 2H), 3.70 (s, 3H), 3.98 (m, 1H), 4.07 (m, 1H), 4.89 (dd, *J* = 6.5, 6.0 Hz, 1H), 5.25 (brs, 1H), 6.29 (d, *J* = 15.5 Hz, 1H), 6.84 (ddd, *J* = 15.5, 8.0, 1.0 Hz, 1H); ECD (MeOH) λ_{max} nm ($\Delta\varepsilon$) 230 (+9.03), 297 (−2.51), 340 (0) (Supplementary Figure S20).

Esterification of 2

To a stirred solution of **2** (1.0 mg, 2.3 μmol) in benzene-MeOH (0.2 ml, 4:1) was added 2.0 M solution of $TMSCHN_2$ in diethyl ether (2.3 μl, 4.6 μmol) at room temperature. The reaction mixture was stirred for 10 min and then concentrated to provide methyl ester **7** (1.0 mg, 95%).

7: ECD (MeOH) λ_{max} nm ($\Delta\varepsilon$) 231 (+8.06), 296 (−2.10), 340 (0) (Supplementary Figure S20).

Acid hydrolysis of 1 and preparation of FDLA derivative

A mixture of **1** (2.4 mg, 5.2 μmol) and 6 N HCl (100 μl) was heated at 110 °C for 18 h in a sealed ampule. After cooling to room temperature, lipophilic

materials were removed by extraction with CHCl₃. The aqueous layer was dried up and dissolved in 100 μl of H₂O. The resulting solution (50 μl) was treated with 50 μl of 1% ʟ-FDLA in acetone and 50 μl of 1 ᴍ NaHCO₃. The mixture was heated at 40 °C for 1.5 h. After cooling to room temperature, the reaction mixture was neutralized with 50 μl of 1 ɴ HCl, and the resulting mixture was added to 300 μl of MeOH to total volume of 500 μl. From the solution, 200 μl aliquot was withdrawn and dried up, and then redissolved in 200 μl of MeOH. The solution (10 μl) was analyzed by LC/MS on a Waters 600E pump system connected to Waters ZQ.

Preparation of FDLA derivatives of *N*-methyl-ʟ-serine and *N*-methyl-ᴅ-serine

Authentic samples of *N*-methyl-ʟ-serine and *N*-methyl-ᴅ-serine were prepared from *N*-cbz-ʟ-serine and *N*-cbz-ᴅ-serine, respectively, according to the procedure reported by Hughes and colleagues.[25] *N*-methyl-ᴅ-serine or *N*-methyl-ʟ-serine was dissolved in H₂O by 1% (w/v), and each of 50 μl aliquot was treated for LC/MS as the same manner as that of hydrolysate of **1**.

Computer calculation of ECD spectrum of compound 2

The conformational analysis and calculation of ECD spectra were performed with Spartan '16 (Wave function, Irvine, CA, USA) on Mac Pro Apple (Early 2008) and Gaussian 09 revision E.01 (Gaussian, Wallingford, CT, USA)[26] on HOKUSAI GreatWave in RIKEN (Wako, Japan). Wakodecaline B (**2**) was submitted to a conformational search employing Merck molecular force field to afford 123 stable conformers. Each conformer was optimized by Hartree–Fock/STO-3G with Spartan '16 and further optimized by density functional theory at B3LYP/6-31G (d) level with Gaussian 09. The optimized conformers were subjected to a time-dependent density functional theory at ωB97XD/6-31G (d) level with Gaussian 09 to obtain calculated ECD spectra. They were averaged based on the Boltzmann distribution of the 5 most stable conformers that were selected with >1.0% of Boltzmann distribution and covered 92.0% of population (see Supplementary Information).

Cytotoxicity, antimicrobial activity and antimalarial tests

The *in vitro* cytotoxicity assay methods against HeLa, HL60 and *src*ᵗˢ-NRK cells have been described in the previous report.[26] The microdilution assay against *S. aureus* 209, *E. coli* HO141, *A. fumigatus* Af293, *P. oryzae* kita-1 and *C. albicans* JCM1542 have been in the previous report.[27] The antimalarial test against *P. falciparum* 3D7 has been described in the previous report.[28]

DEDICATION
This article is dedicated to the special issue for Professor Hamao Umezawa.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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