The Journal of Antibiotics (2013) 66, 23–29

www.nature.com/ja

ORIGINAL ARTICLE

A new ascochlorin derivative from *Cylindrocarpon* sp. FKI-4602

Mio Kawaguchi[1], Takashi Fukuda[1], Ryuji Uchida[1], Kenichi Nonaka[2], Rokuro Masuma[2] and Hiroshi Tomoda[1]

Cylindrol A_5, a new ascochlorin congener, was isolated along with 14 known compounds from the culture broth of *Cylindrocarpon* sp. FKI-4602 by solvent extraction, octadecylsilane column chromatography and HPLC. The structure of cylindrol A_5 was elucidated by spectral analyses, including NMR. The compound has an ascochlorin skeleton consisting of a resorcin aldehyde and a cyclohexanone moieties. Cylindrol A_5 showed moderate antimicrobial activity against *Bacillus subtilis*, *Kocuria rhizophila*, *Mycobacterium smegmatis* and *Acholeplasma laidlawii*. The biosynthetic pathway to cylindrol A_5 was deduced from the 14 isolated metabolites of the fungal strain.
The Journal of Antibiotics (2013) **66,** 23–29; doi:10.1038/ja.2012.75; published online 21 November 2012

Keywords: ascochlorin derivative; *Cylindrocarpon* sp.; cylindrol A_5; fungal metabolite

INTRODUCTION

Filamentous fungi have the potential to produce a variety of bioactive compounds and are recognized as a rich source for new drug discovery.[1,2] Recent advances in genomic analysis have revealed that filamentous fungi such as *Aspergillus niger* possess about 80 biosynthetic gene clusters for secondary metabolites produced via pathways of polyketide synthase or non-ribosomal peptide synthase.[3]

In the previous study, we reported an efficient method for isolating antifungal antibiotics producing fungi from soil samples, and obtained 26 candidate fungi from 18 soil samples.[4] Among them, strain FKI-4602 was considered to belong to the genus *Cylindrocarpon*. The number of secondary metabolites of *Cylindrocarpon* was 39 from Chapman & Hall Chemical Database,[5] which was much fewer than those of *Aspergillus* sp. (1536), *Fusarium* sp. (477), *Penicillium* sp. (1449) and *Trichoderma* sp. (274). These findings prompted us to carry out comprehensive analysis of the secondary metabolites of *Cylindrocarpon* sp. FKI-4602. This fungus was found to produce about 20 compounds by HPLC analysis. Among them, 15 compounds were purified from the culture broth of the fungi; 14 known compounds including ascochlorin,[6] ilicicolin E[6] and ilicicolin F[6] with antifungal activity, and a new ascochlorin congener named cylindrol A_5 (Figure 1).

In this study, the taxonomy of fungal strain FKI-4602, structure elucidation and biological activity of cylindrol A_5 are described. Furthermore, the biosynthetic pathway of cylindrol A_5 is deduced from the structures of 14 compounds produced by the fungus.

RESULTS

Taxonomic study of strain FKI-4602

Colonies on potato dextrose agar (PDA) after 7 days at 25 °C (Figure 2a) were 26–28 mm in diameter, floccose, planar with white (a) aerial mycelia, and exuding sparse clear drops. The reverse side was pale brown (4 gc) with an entire margin, without soluble pigment. Colonies on Miura's medium (LcA) after 7 days at 25 °C (Figure 2b), were 34–35 mm in diameter, planar with white (a) floccose aerial mycelia and exuding sparse clear drops. The reverse side was white (a) with an entire margin, without soluble pigment. Colonies on potato carrot agar (PCA) after 7 days at 25 °C (Figure 2c) were 37–38 mm in diameter, planar with white (a) floccose aerial mycelia and exuding sparse clear drops. The reverse side was white (a) with an entire margin, without soluble pigment. Colonies on PDA at 5 and 37 °C showed no growth. Macroconidia were cylindrical, straight or slightly curved with bluntly rounded ends and 3–5 septate, and 32.5–52.5 × 2.5–5.5 μm in size (Figure 3).

The total length of the rDNA internal transcribed spacer (ITS; including 5.8S rDNA) of FKI-4602 was 588 base pairs in a BLAST search using BLASTN 2.2.26 from the National Center for Biotechnology Information (NCBI).[7] FKI-4602 had 94.4% similarity (28 nucleotides are different) with the nucleotide sequences of *Cylindrocarpon olidum* var. *crassum* CBS 216.67 (GenBank accession number AY677294).

From these results of morphological characteristics[8] and BLAST search, strain FKI-4602 was considered to belong to the genus *Cylindrocarpon*.

Isolation

The isolation procedure is shown in Figure 4. Six-day-old culture broth (5.0 l) of *Cylindrocarpon* sp. FKI-4602 was centrifuged. The supernatant (2.2 l) was adjusted to pH 3 with HCl and extracted with ethyl acetate (4.4 l). The organic layer was dried with Na_2SO_4 and concentrated *in vacuo* to dryness to yield an oily material (1.7 g). Part of the material (600 mg) was subjected to centrifugal partition

[1]Graduate School of Pharmaceutical Sciences, Kitasato University, Tokyo, Japan and [2]Kitasato Institute for Life Sciences, Kitasato University, Tokyo, Japan
Correspondence: Professor H Tomoda, Graduate School of Pharmaceutical Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan.
E-mail: tomodah@pharm.kitasato-u.ac.jp
Received 19 June 2012; revised 16 August 2012; accepted 21 August 2012; published online 21 November 2012

chromatography (System Instruments, Tokyo, Japan) under the following conditions: a two-phase solvent system, upper and lower layers of chloroform–methanol–water (2:2:1) as stationary and mobile phases, respectively; flow rate, 3 ml min^{-1}; rotation speed, 700 r.p.m. The upper layer of the solvent was introduced by the ascending method (480 ml). Three fractions A to C were then collected and concentrated under reduced pressure. Fraction A (7.3 mg) was purified by HPLC; HPLC conditions were as follws: octadecylsilane (ODS) column (20 × 250 mm; Pegasil, Senshu Scientific, Tokyo, Japan), isocratic 20% CH_3CN, flow rate 8 ml min^{-1} and detected at 210 nm. Under these conditions, orcinol (**2**)[9] was eluted (3.0 mg, retention time (Rt) 12.0 min). Fraction B (12.3 mg) was purified by HPLC; ODS column (20 × 250 mm), isocratic 30% CH_3CN in 0.05% H_3PO_4, flow rate 8 ml min^{-1} and detected at 210 nm. Under these conditions, orsellinic acid (**3**)[10] was eluted (6.2 mg, Rt 7.8 min). Fraction C (376.0 mg) was purified by HPLC; ODS column (20 × 250 mm), isocratic 25-min 75% CH_3CN in 0.05% H_3PO_4, then 15-min linear gradient from 75 to 100% CH_3CN in 0.05% H_3PO_4, flow rate 8 ml min^{-1} and detected at 210 nm. A typical isolation profile of **1** and **4** to **15** is shown in Figure 4. Under these conditions, 13 compounds were eluted: cylindrol B (**4**)[11] (3.5 mg, Rt 18.4 min), LL-Z 1272ε (**5**)[6] (10.7 mg, Rt 20.8 min), ilicicolin F (**6**)[6,12] (3.5 mg, Rt 22.4 min), ilicicolin E (**7**)[6,12] (1.7 mg, Rt 27.2 min), ascochlorin (**8**)[6] (13.0 mg, Rt 30.4 min), ilicicolin C (**9**)[6,12] (7.5 mg, Rt 34.4 min), linolenic acid (**10**)[13] (2.0 mg, Rt 40.0 min), cylindrol A_4 (**11**)[11] (2.0 mg, Rt 40.8 min), LL-Z 1272 β (**12**)[6] (4.0 mg, Rt 44.0 min), linoleic acid (**13**)[13] (53.8 mg, Rt 45.6 min), LL-Z 1272α (**14**)[14] (2.6 mg, Rt 50.4 min) and oleic acid (**15**)[13] (37.3 mg, Rt 52.8 min). Cylindrol A_5 (**1**) was eluted as a peak with a retention time of 36.8 min (Figure 5). The fraction of the peak was pooled and concentrated to yield pure **1** (4.6 mg) as a pale brown powder.

Structure elucidation of cylindrol A_5

Physico-chemical properties of cylindrol A_5 (**1**) are summarized in Table 1. Compound **1** showed absorption maxima at 228, 293 and 344 nm in the UV spectrum. Compound **1** showed characteristic absorption at 3433, 1727 and 1705 cm^{-1} in the IR spectrum, suggesting the presence of a hydroxy, an ester and an aldehyde groups.

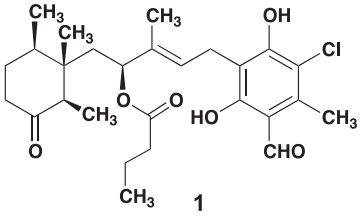

Figure 1 Structure of cylindrol A_5 (**1**).

The molecular formula of **1** was determined to be $C_{27}H_{37}ClO_6$ on the basis of HR-ESI-MS measurement (Table 1). The ^{13}C-NMR spectrum (in $CDCl_3$) showed 27 resolved signals (Table 2). They were classified into 6 methyl carbons, 6 methylene carbons, 5 methine carbons, including two sp^2 methine carbons, and 10 quaternary carbons, including 9 sp^2 quaternary carbons by analysis of the DEPT spectra. The ^{1}H and ^{13}C NMR along with the HSQC spectrum revealed the assignments of these signals as shown in Table 2. Analysis of the ^{1}H–^{1}H COSY spectrum and ^{13}C–^{1}H long-range couplings of ^2J and ^3J observed in the HMBC spectrum gave the four partial structures A to D, as shown in Figure 6; (A) The cross peaks from 15-H (δ 1.93) to C-14 (δ 44.0), from 17-H_2 (δ 2.20, 2.26) to C-18 (δ 213.3) and C-19 (δ 50.4), from 19-H (δ 2.54) to C-14, C-15 (δ 36.6) and C-18, from 20-H_3 (δ 0.54) to C-14, C-15 and C-19, from 21-H_3 (δ 0.95) to C-14, and from 22-H_3 (δ 0.80) to C-14 and C-18 supported the partial structure A. (B) The cross peaks from 9-H_2 (δ 3.37, 3.41) to C-11 (δ 135.5), from 10-H (δ 5.54) to C-23 (δ 11.9), from 12-H (δ 5.37) to C-10 (δ 124.5), C-11 and C-23, and from 23-H_3 (δ 1.80) to C-10, C-11 and C-12 (δ 75.6) supported the partial structure B. (C) The cross peaks from 2-OH (δ 12.66) to C-1 (δ 113.6), C-2 (δ 162.2) and C-3 (δ 113.6), from 4-OH (δ 6.32) to C-3, C-4 (δ 156.0) and C-5 (δ 113.3), from 7-H_3 (δ 2.59) to C-1, C-5 and C-6 (δ 137.9), and from 8-H (δ 10.12) to C-1 and C-2 supported the partial structure C. (D) The cross peaks from 25-H_2 (δ 2.24) to C-24 (δ 172.7), and from 26-H_2 (δ 1.63) to C-24, supposed the partial structure D. The cross peaks from 12-H to C-14 and C-24, from 13-H_2 (δ 1.52, 1.82) to C-14, C-19 and C-20 (δ 15.4), and from 20-H_3 to C-13 (δ 39.6) observed in the HMBC spectra indicated that the partial structures A, B and D were linked, as shown in Figure 7. In addition, the cross peaks from 9-H_2 to C-3 and C-4, and from 10-H to C-3 indicated that the partial structures B and C were linked, as shown in Figure 7. The chloride atom at C-5 was elucidated from the chemical shift and MS spectrum. The structure satisfied the molecular formula and the degrees of unsaturation. Taken together, the planar structure of **1** was elucidated as shown in Figure 7.

The relative stereochemistry at C-12, C-14, C-15 and C-19 was investigated by a ROESY experiment. NOE correlations were observed between 12-H and 13-H, 12-H and 19-H, 13-H and 15-H, 13-H and 20-H, 20-H and 21-H, and 20-H and 22-H (Figure 8). Comparing the NOE data of **1** with those of known vertihemipterin,[15] the relative stereochemistry of **1** was presumed to be 12*R**14*S**15*R**19*R** (Figure 1).

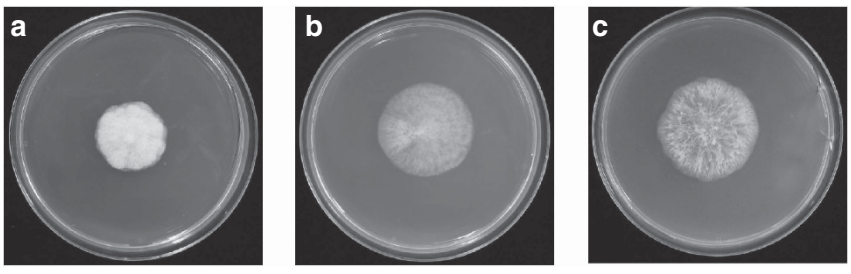

Figure 2 Morphological characteristics of *Cylindrocarpon* sp. FKI-4602. (**a**) Photograph of colonies grown on potato dextrose agar after 7 days. (**b**) Photograph of colonies grown on LcA after 7 days. (**c**) Photograph of colonies grown on potato carrot agar after 7 days. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

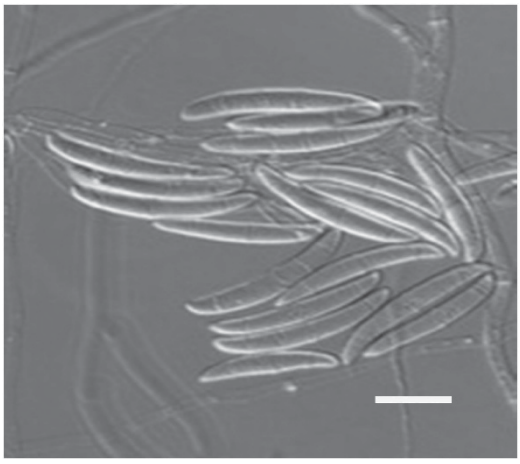

Figure 3 Micrograph of macroconidia grown on LcA. Scale bar, 20 μm. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

Antimicrobial activity

Cylindrol A_5 (**1**) showed moderate antimicrobial activity against *Bacillus subtilis*, *Kocuria rhizophila*, *Mycobacterium smegmatis* and *Acholeplasma laidlawii*, with inhibition zones of 7, 8, 8 and 8 mm at 10 μg per 6 mm disk, respectively. Ascochlorin (**8**) showed moderate antimicrobial activity against *B. subtilis*, *M. smegmatis* and *Candida albicans* with inhibition zones of 7, 7 and 10 mm at 10 μg per 6 mm disk, respectively.

DISCUSSION

Fungal strain FKI-4602, identified as the genus *Cylindrocarpon* in this study, was recently isolated by our method for directly isolating antibiotic-producing fungi from soil samples.[4] In this method, antagonistic effects were directly observed on agar plates between fungal colonies from soil samples and *C. albicans* overlaid by spraying. *Cylindrocarpon* species were reported to be isolated from the bark of woody plants or decaying herbaceous materials in tropical or temperate regions.[16]

The genus of *Cylindrocarpon* consists of about 70 species,[17] and the number of secondary metabolites from this genus is only 39,[5] including cylindrol B,[11] cyclosporine[18] and radicicol.[19]

In this study, comprehensive analysis of the metabolites produced by *Cylindrocarpon* sp. FKI-4602 was carried out. As demonstrated, 15 compounds including a new ascochlorin congener cylindrol A_5 (**1**) were obtained from the culture broth. From their antimicrobial

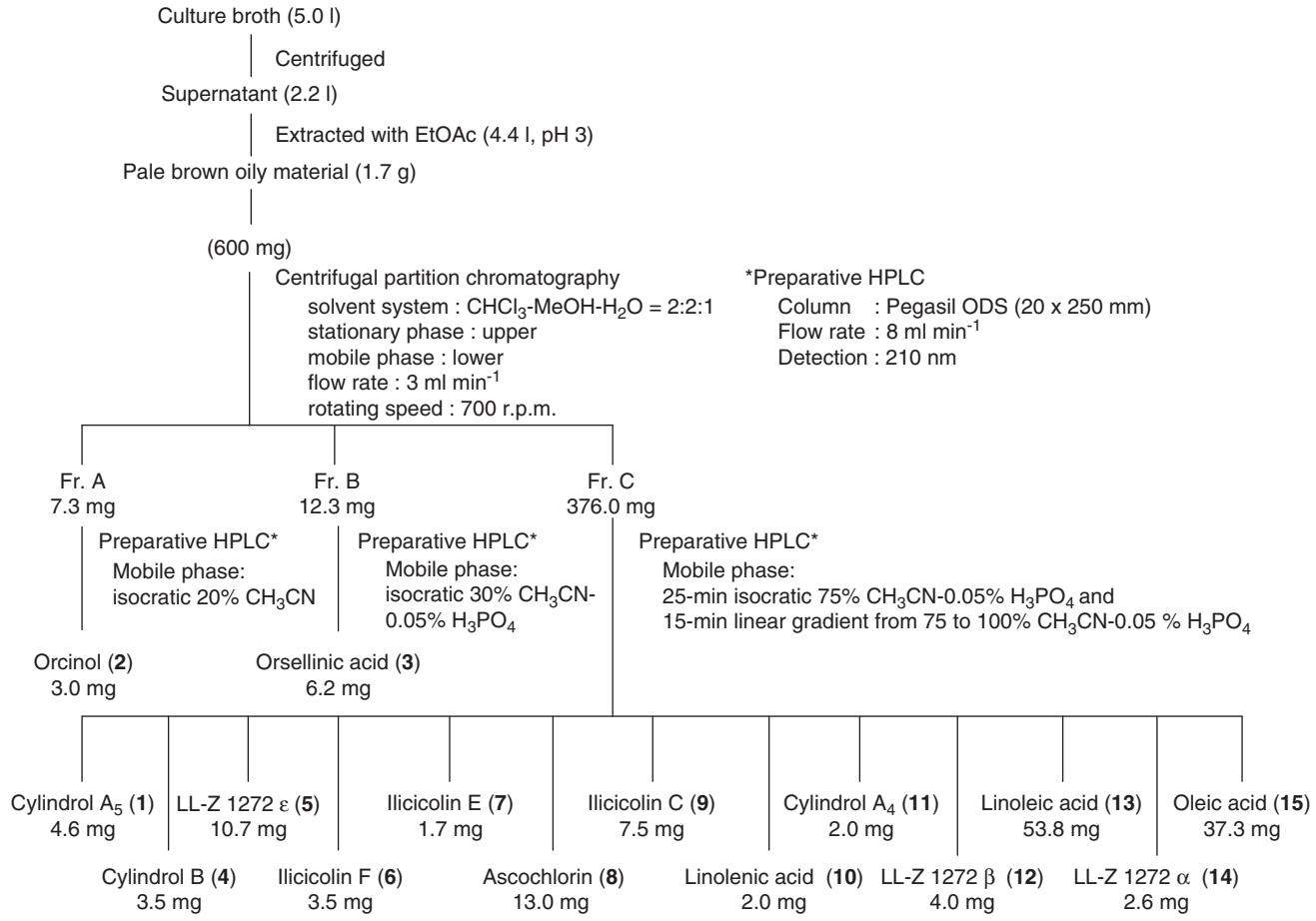

Figure 4 Isolation procedure of metabolites from the culture broth of *Cylindrocarpon* sp. FKI-4602.

activity, ilicicolin F (**6**), ilicicolin E (**7**) and ascochlorin (**8**) showed anti-*C. albicans* activity with inhibitory zones of 23, 11 and 15 mm at a concentration of 10 μg per 6 mm paper disk, respectively. Thus, we confirmed that fungus FKI-4602 produced anti-*C. albicans* antibiotics **6**–**8**. As **1,** cylindrol B (**4**) and cylindrol A_4 (**11**) showed no antifungal activity, the double bond between C-12 and C-13, and a chloride atom might be necessary to show antifungal activity.

From the 15 metabolites produced by *Cylindrocarpon* sp. FKI-4602, possible biosynthetic correlations among them are summarized in Figure 9. A fatty acid, oleic acid (**15**), is biosynthesized from the acetate–malonate (fatty acid or polyketide) pathway, then sequentially converted to linoleic acid (**13**) and linolenic acid (**10**; Group 1). Similarly, orcellinic acid (**3**) is biosynthesized from this pathway and decarboxylated to yield orcinol (**2**; Group 2). Hunter and Mellows[20] reported that **8** was biosynthesized through the mevalonate and polyketide pathways. The farnesyl residue from the mevalonate pathway is cyclized and conjugated with the orsellinic aldehyde from the polyketide to produce **8**. In this study, the key intermediates **8**, LL-Z 1272 β (**12**) and LL-Z 1272 α (**14**) were identified (Groups 3 and 4). Finally, **1** and **11** are produced by hydration and acylation from **8** (Group 5).

Ten ascochlorin congeners had been already reported to be isolated from *Cylindrocarpon* species.[11,21] In this study, five more ascochlorin congeners, including the new cylindrol A_5, were identified as metabolites of *Cylindrocarpon* sp. FKI-4602. These findings suggest that the genus *Cylindrocarpon* has the ascochlorin biosynthetic pathway in common.

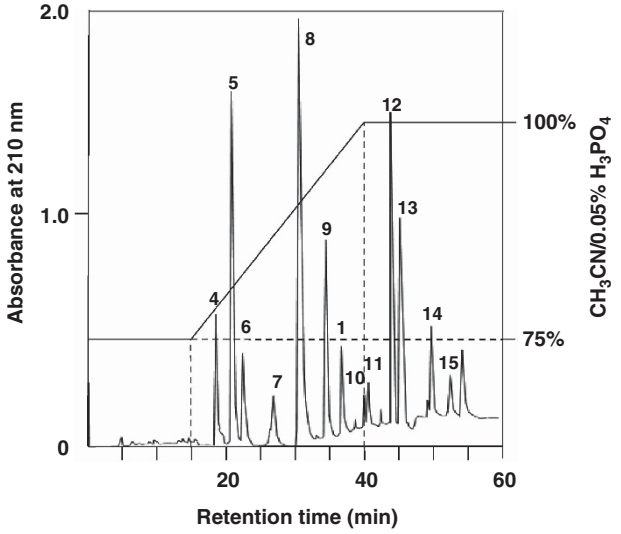

Figure 5 Chromatographic profile of cylindrol A_5 purification by preparative HPLC. HPLC conditions: column, PEGASIL ODS (20 × 250 mm); solvent, 25-min isocratic of 75% CH_3CN in 0.05% H_3PO_4 and 15-min linear gradient from 75 to 100% CH_3CN in 0.05% H_3PO_4; detection at 210 nm; flow rate, 8 ml min^{-1}; sample, 130 mg of materials (obtained through centrifugal partition chromatography) dissolved in 6.5 ml methanol; injection 100 μl.

Table 1 Physico-chemical properties of cylindrol A_5

	1
Appearance	Pale brown powder
Molecular weight	492
Molecular formula	$C_{27}H_{37}ClO_6$
HRESI-TOF-MS (m/z)	
Calcd	491.2224 $[M-H]^-$
Found	491.2200 $[M-H]^-$
UV (MeOH) λ_{max} nm (log ε)	228 (4.06), 293 (3.84), 344 (3.61)
$[\alpha]_D^{25}$	+15.7°(c 0.1, MeOH)
IR (KBr) ν_{max} $(cm)^{-1}$	3433, 2925, 1727, 1705, 1631, 1460

MATERIALS AND METHODS

General experimental procedure

Optical rotation was recorded with a DIP-370 digital polarimeter (Jasco, Tokyo, Japan). ESIMS spectrometry was conducted using a JMS-T100LP spectrometer (JEOL, Tokyo, Japan). UV and IR spectra were measured with a

Table 2 ^{13}C and ^{1}H NMR chemical shift of cylindrol A_5

	1	
No.	δ_C[a]	δ_H (*J* in Hz)[b]
1	113.6	—
2	162.2	—
3	113.6	—
4	156.0	—
5	113.3	—
6	137.9	—
7	14.5	2.59 s
8	193.2	10.12 s
9	21.6	3.37 dd (7.5,14.5)
		3.41 dd (7.5,14.5)
10	124.5	5.54 t (7.5)
11	135.5	—
12	75.6	5.37 dd (4.0,7.5)
13	39.6	1.52 dd (4.0,16.0)
		1.82 dd (7.5,16.0)
14	44.0	—
15	36.6	1.93 m
16	31.1	1.79 m
		1.54 m
17	41.5	2.20 m
		2.26 m
18	213.3	—
19	50.4	2.54 q (6.5)
20	15.4	0.54 s
21	15.6	0.95 d (7.0)
22	7.9	0.80 d (6.5)
23	11.9	1.80 s
24	172.7	—
25	36.6	2.24 m
26	18.4	1.63 m
27	13.6	0.91 t (7.5)
2-OH		12.66 s
4-OH		6.32 s

[a]Chemical shifts are shown with reference to $CDCl_3$ as 77.0 p.p.m.
[b]Chemical shifts are shown with reference to $CDCl_3$ as 7.26 p.p.m.

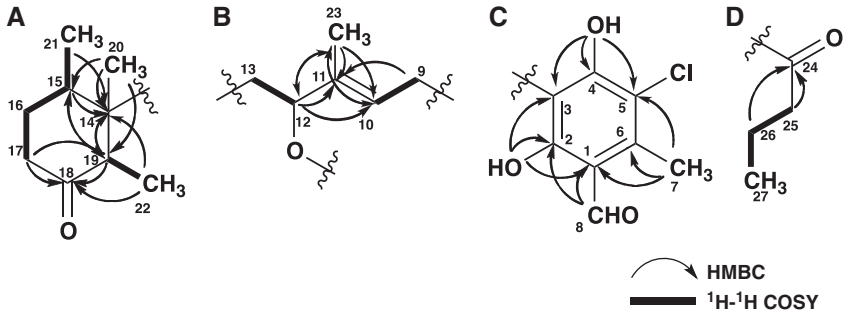

Figure 6 Partial structures (**A**) to (**D**) of cylindrol A_5.

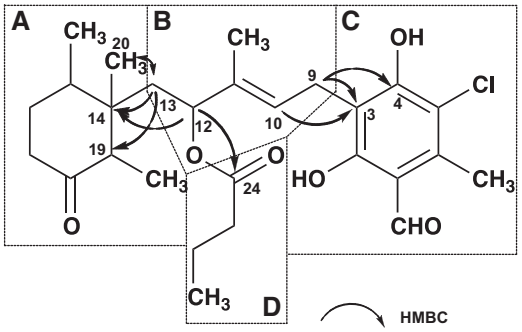

Figure 7 Key correlations of cylindrol A_5 in ^{13}C-^{1}H HMBC experiments.

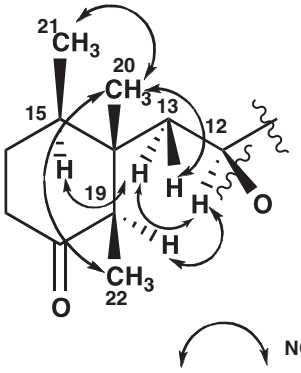

Figure 8 Key cross peaks of cylindrol A_5 in ROESY experiments.

DU640 spectrophotometer (Beckman Coulter, Brea, CA, USA) and FT-210 Fourier transform intrated spectrometer (HORIBA, Kyoto, Japan), respectively. The various NMR spectra were measured with Mercury 300 MHz, NMR System 400 MHz or INOVA 600 MHz spectrometer (Agilent Technology, Santa Clara, CA, USA).

Taxonomic studies

Fungal strain FKI-4602 was isolated from soil collected in Hakone, Kanagawa, Japan, in 2007. For determination of the morphological characteristics, the isolates were inoculated as 1-point cultures on PDA (Becton Dickinson, Sparks, MD, USA), LcA (glycerol 0.10%, KH_2PO_4 0.08%, K_2HPO_4 0.02%, $MgSO_4 \cdot 7H_2O$ 0.02%, KCl 0.02%, $NaNO_3$ 0.2%, yeast extract 0.02% and agar 1.5%, adjusted to pH 6.0 before sterilization) and PCA[22] (potato 2.0%, carrot 2.0% and agar 2.0%), and then grown at 25 °C (also at 5 and 37 °C on PDA) for 7 days in the dark. The Color Harmony Manual 4th Edition[23] was used to determine color names and hue numbers. For the determination of micro-morphological characteristics, the samples were observed under a Vanox-S AH-2 microscope (Olympus, Tokyo, Japan). Genomic DNA of fungal strain FKI-4602 was isolated using the PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. The rDNA ITS, including 5.8S rDNA, was amplified using primers ITS1 and ITS4[24] in a PCR thermal cycler Dice mini Model TP100 (TaKaRa, Shiga, Japan), and the PCR products were purified using a QIAquick, PCR DNA Purification kit (Qiagen, Valencia, CA, USA). The PCR products were sequenced directly in both directions using primers ITS1, ITS2, ITS3 and ITS4, using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing products were purified by ethanol/EDTA precipitation, and samples were analyzed on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). Contigs were assembled using the forward and reverse sequences with the SeqMan and SeqBuilder programs from the Lasergene7 package (DNASTAR, Madison, WI, USA). The ITS sequence of the strain was deposited in the DDBJ with accession number AB725901 for FKI-4602.

Fermentation

The strain was grown on an LcA slant (7.5 ml, in a glass screw-cap tube, 16 × 150 × 9 mm) at 25 °C for 14 days. The seed medium (glucose 2.0%, yeast extract 0.2%, $MgSO_4 \cdot 7H_2O$ 0.05%, polypeptone (Nihon Pharmaceutical, Tokyo, Japan) 0.5%, KH_2PO_4 0.1% and agar 0.1%, adjusted to pH 6.0 before sterilization) was used for inoculation. The flask was shaken on a rotary shaker at 27 °C for 3 days. The seed culture (100 ml) was transferred into fifty 500-ml Erlenmeyer flasks containing 100 ml production medium (glucose 1.0%, soluble starch 2.0%, soybean oil 2.0%, Pharmamedia (Sanko Junyaku, Tokyo, Japan) 1.0%, bonito extract (Kyokuto Pharmaceutical Industrial, Tokyo, Japan) 0.5%, $MgSO_4 \cdot 7H_2O$ 0.1%, $CaCO_3$ 0.3%, $FeSO_4 \cdot 7H_2O$ 0.0001%, $MnCl_2 \cdot 4H_2O$ 0.0001%, $ZnSO_4 \cdot 7H_2O$ 0.0001%, $CuSO_4 \cdot 5H_2O$ 0.0001%, $CoCl_2 \cdot 6H_2O$ 0.0001% and agar 0.1%, adjusted to pH 6.0 before sterilization). Fermentation was carried out at 27 °C for 6 days with agitation at 210 r.p.m.

Metabolite identification

Cylindrol A_5 (**1**): NMR spectral data (600 MHz, $CDCl_3$) are presented in Table 2.

Orcinol (**2**): ^{1}H NMR (400 MHz, CD_3OD) δ: 2.17 (3H, s), 6.06 (1H, t, $J = 2.2$ Hz), 6.12 (2H, d, $J = 2.2$ Hz), EIMS (*m/z*): 124 $[M]^+$, molecular formula: $C_7H_8O_2$. These data were identical to those reported previously.[9]

Orsellinic acid (**3**): ^{1}H NMR (300 MHz, CD_3OD) δ: 2.49 (3H, s), 6.14 (1H, d, $J = 2.5$ Hz), 6.19 (1H, d, $J = 2.5$ Hz), EIMS (*m/z*):168 $[M]^+$, molecular formula: $C_8H_8O_4$. These data were identical to those reported previously.[10]

Cylindrol B (**4**): ^{1}H NMR (300 MHz, $CDCl_3$), δ: 0.70 (3H, s), 0.82 (3H, d, $J = 6.6$ Hz), 0.84 (3H, d, $J = 6.6$ Hz), 1.60–1.75 (2H, m), 1.88–1.98 (1H, m), 1.94 (3H, s), 2.28–2.46 (2H, m), 2.40 (1H, m), 2.50 (3H, s), 3.51 (2H, t, $J = 7.3$ Hz), 5.42 (1H, d, $J = 16.0$ Hz), 5.51 (1H, t, $J = 7.2$ Hz), 5.72 (1H, brs), 5.92 (1H, d, $J = 16.0$ Hz), 6.20 (1H, s), 10.09 (1H, s), 12.73 (1H, s), ESIMS (*m/z*): 369 $[M\text{-}H]^-$, molecular formula: $C_{23}H_{30}O_4$. These data were identical to those reported previously.[11]

LL-Z 1272ε (**5**) ^{1}H NMR (300 MHz, $CDCl_3$) δ: 0.58 (3H, s), 0.88 (3H, d, $J = 6.4$ Hz), 0.92 (3H, d, $J = 6.4$ Hz), 1.32–1.41 (2H, m), 1.60–1.68 (1H, m),

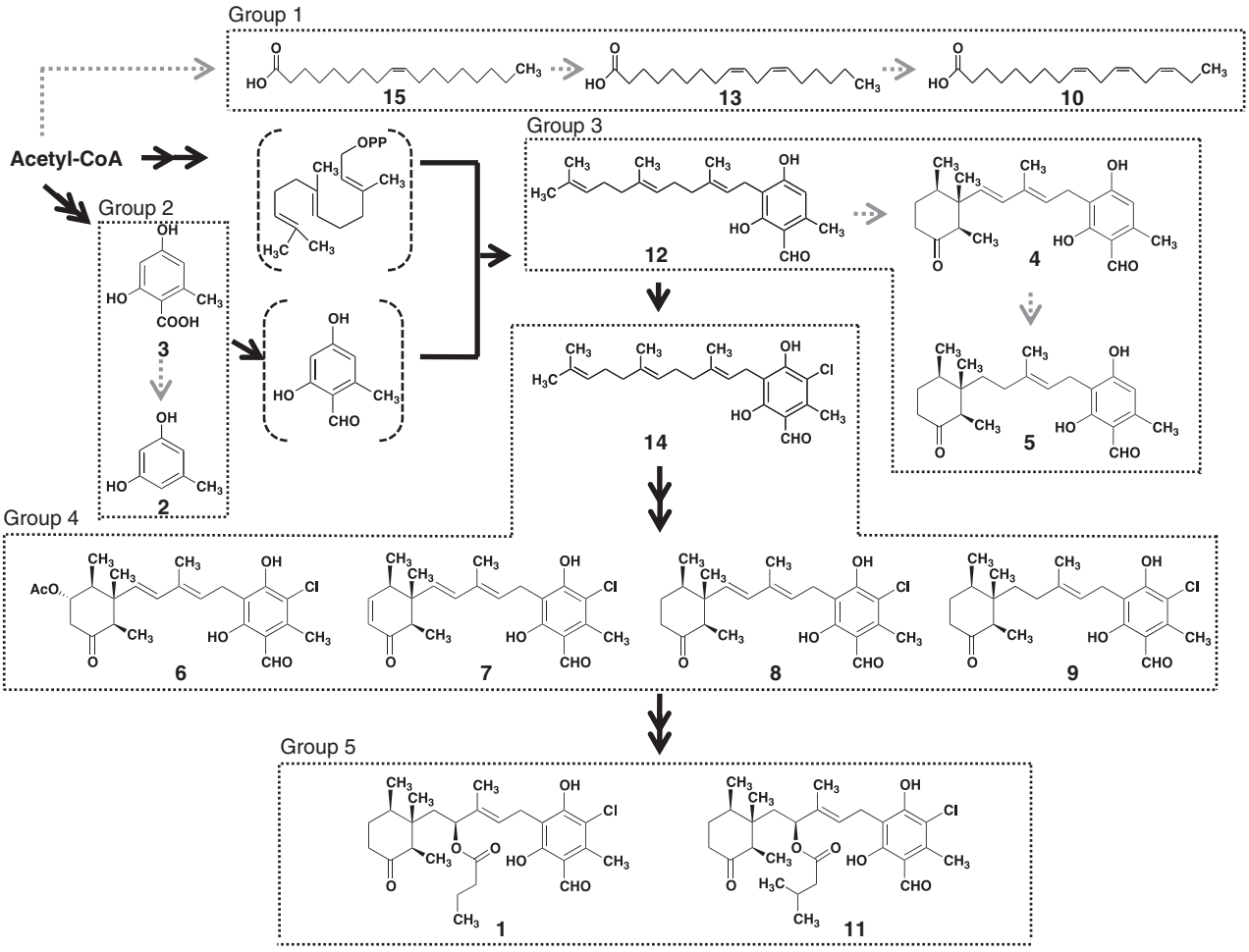

Figure 9 Possible biosynthetic pathway to cylindrol A_5.

1.84 (3H, s), 1.86–2.11 (4H, m), 2.34 (2H, m), 2.47 (1H, m), 2.50 (3H, s), 3.40 (2H, d, $J=7.0$ Hz), 5.28 (1H, t, $J=7.3$ Hz), 6.01 (1H, brs), 6.21 (1H, s), 10.08 (1H, s), 12.76 (1H, s), ESIMS (*m/z*): 371 $[M\text{-}H]^-$, molecular formula: $C_{23}H_{32}O_4$. These data were identical to those reported previously.[6]

Ilicicolin F (**6**): ^1H NMR (300 MHz, $CDCl_3$) δ: 0.73 (3H, s), 0.87 (6H, d, $J=6.6$ Hz), 1.92 (3H, s), 1.99 (1H, m), 2.06 (3H, s), 2.42 (1H, m), 2.50 (1H, m), 2.61 (3H, s), 2.87 (1H, dd, $J=5.8$, 13.2 Hz), 3.54 (2H, d, 7.3 Hz), 4.89 (1H, ddd, $J=5.9$, 11.0, 11.5 Hz), 5.32 (1H, d, $J=15.3$ Hz), 5.55 (1H, t, $J=8.0$ Hz), 5.92 (1H, d, $J=15.3$ Hz), 6.37 (1H, brs), 10.15 (1H, s), 12.71 (1H, brs), ESIMS (*m/z*): 461 $[M\text{-}H]^-$, molecular formula: $C_{25}H_{31}ClO_6$. These data were identical to those reported previously.[6,12]

Ilicicolin E (**7**): ^1H NMR (300 MHz, $CDCl_3$) δ: 0.79 (3H, s), 0.94 (3H, d, $J=6.6$ Hz), 0.97 (3H, d, $J=7.8$ Hz), 1.93 (3H, brs), 2.46 (1H, q, $J=6.6$ Hz), 2.60 (3H, s), 2.62 (1H, m), 3.54 (2H, d, $J=7.6$ Hz), 5.42 (1H, d, $J=15.8$ Hz), 5.54 (1H, t, $J=7.8$ Hz), 5.94–6.05 (2H, m), 6.37 (1H, brs), 6.55 (1H, dd, $J=2.1$, 10.3 Hz,), 10.15 (1H, s), 12.71 (1H, s), ESIMS (*m/z*): 401 $[M\text{-}H]^-$, molecular formula: $C_{23}H_{27}ClO_4$. These data were identical to those reported previously.[6,12]

Ascochlorin (**8**): ^1H NMR (300 MHz, $CDCl_3$) δ: 0.69 (3H, s), 0.80 (3H, d, $J=6.6$ Hz), 0.83 (3H, d, $J=6.6$), 1.63 (1H, m), 1.86–1.98 (2H, m), 1.92 (3H, brs), 2.38 (3H, m), 2.60 (3H, s), 3.53 (2H, d, $J=7.3$ Hz), 5.37 (1H, d, $J=16.1$ Hz), 5.52 (1H, t, $J=7.8$ Hz), 5.89 (1H, d, $J=16.1$ Hz), 6.37 (1H, brs), 10.14 (1H, s), 12.71 (1H, s), ESIMS (*m/z*): 403 $[M\text{-}H]^-$, molecular formula: $C_{23}H_{29}ClO_4$. These data were identical to those reported previously.[6]

Ilicicolin C (**9**): ^1H NMR (300 MHz, $CDCl_3$) δ: 0.55 (3H, s), 0.87 (3H, d, $J=6.7$ Hz), 0.90 (3H, d, $J=6.7$ Hz), 1.38 (2H, dt, $J=5.4$, 11.4 Hz), 1.64 (1H, m), 1.76–1.90 (2H, m), 1.81 (2H, s), 1.98 (1H, m), 2.32 (2H, m), 2.44 (3H, q, $J=5.9$ Hz), 2.60 (3H, s), 3.39 (2H, d, $J=7.2$ Hz), 5.24 (1H, t, $J=7.2$ Hz), 6.38 (1H, brs), 10.14 (1H, s), 12.69 (1H, s), ESIMS (*m/z*): 405 $[M\text{-}H]^-$, molecular formula: $C_{23}H_{31}ClO_4$. These data were identical to those reported previously.[6,12]

Linolenic acid (**10**): ^1H NMR (400 MHz, $CDCl_3$) δ: 0.80 (3H, t, $J=7.6$ Hz), 1.30–1.38 (8H, m), 1.64 (2H, m), 2.02–2.10 (4H, m), 2.35 (2H, t, $J=7.5$ Hz), 2.81 (4H, t, $J=5.8$ Hz), 5.36 (6H, m), ESIMS (*m/z*): 277 $[M\text{-}H]^-$, molecular formula: $C_{18}H_{30}O_2$. These data were identical to those reported previously.[13]

Cylindrol A_4 (**11**): ^1H NMR (300 MHz, $CDCl_3$) δ: 0.54 (3H, s), 0.81 (3H, d, $J=6.9$ Hz), 0.91 (6H, d, $J=6.4$ Hz), 0.95 (3H, d, $J=6.7$ Hz), 1.51 (1H, dd, $J=3.6$, 15.3 Hz), 1.56 (2H, m), 1.79 (1H, dd, $J=3.6$, 15.3 Hz), 1.81 (3H, s), 1.96 (1H, m), 2.08 (1H, m), 2.13 (2H, m), 2.19–2.55 (2H, m), 2.54 (1H, q, $J=6.5$ Hz), 2.59 (3H, s), 3.39 (2H, d, $J=7.4$ Hz), 5.36 (1H, m), 5.56 (1H, t, $J=7.3$ Hz), 6.31 (1H, brs), 10.12 (1H, s), 12.66 (1H, s), ESIMS (*m/z*): 505 $[M\text{-}H]^-$, $C_{28}H_{39}ClO_6$. These data were identical to those reported previously.[11]

LL Z-1272 β (**12**): ^1H NMR (300 MHz, $CDCl_3$) δ: 1.59 (6H, s), 1.66 (3H, s), 1.81 (3H, s), 1.95–2.25 (8H, m), 2.49 (3H, s), 3.40 (2H, d, $J=7.0$ Hz), 5.07 (2H, tq, $J=1.3$, 6.4 Hz), 5.26 (1H, t, $J=7.4$ Hz), 6.15 (1H, brs), 6.21 (1H, s), 10.07 (1H, s), 12.76 (1H, s), ESIMS (*m/z*): 355 $[M\text{-}H]^-$, $C_{23}H_{32}O_3$. These data were identical to those reported previously.[6]

Linoleic acid (**13**): ^1H NMR (300 MHz, $CDCl_3$) δ: 0.89 (3H, t, $J=6.8$ Hz), 1.26–1.38 (14H, m), 1.65 (2H, m), 2.05 (4H, dd, $J=6.6$, 13.3 Hz), 2.35 (2H, t, $J=7.5$ Hz), 2.77 (2H, t, $J=5.9$ Hz), 5.35 (4H m), ESIMS (*m/z*): 279 $[M\text{-}H]^+$, molecular formula: $C_{18}H_{32}O_2$. These data were identical to those reported previously.[13]

LL-Z1272 α (**14**): ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.55 (6H, s), 1.64 (3H, s), 1.76 (3H, s), 1.88-2.12 (8H, m), 2.60 (3H, s), 3.40 (2H, d, $J=7.1$ Hz), 5.06 (2H, tq, $J=1.2$, 6.3 Hz), 5.22 (1H, tq, $J=1.0$, 6.9 Hz), 6.42 (1H, brs), 10.14 (1H, s), 12.66 (1H, s), ESIMS (*m/z*): 389 $[M\text{-}H]^-$, $C_{23}H_{31}ClO_3$. These data were identical to those reported previously.[14]

Oleic acid (**15**): ^{1}H NMR (300 MHz, $CDCl_3$) δ: 0.88 (3H, t, $J=6.6$ Hz), 1.26–1.36 (20H, m), 1.63 (2H, m), 2.02 (4H, dd, $J=6.6$, 12.5 Hz), 2.34 (2H, t, $J=7.5$ Hz), 5.34 (2H, m), ESIMS (*m/z*): 281 $[M\text{-}H]^-$, molecular formula: $C_{18}H_{34}O_2$. These data were identical to those reported previously.[13]

Antimicrobial activity

Antimicrobial activity against the following 14 microorganisms was measured by the agar diffusion method using paper discs (6 mm; ADVANTEC, Tokyo, Japan) containing a test sample.[25]

The culture conditions were as follows: *B. subtilis* ATCC 6633 (Davis synthetic medium (K_2HPO_4 0.7%, KH_2PO_4 0.2%, sodium citrate 0.5%, ammonium sulfate 0.1%, glucose 0.2%, $MgSO_4 \cdot 7H_2O$ 0.01% and agar 0.8%), inoculation 1.0%, 37 °C, 24 h); *Staphylococcus aureus* ATCC 6538P (nutrient agar (peptone (Becton Dickinson) 0.5%, meat extract 0.5% and agar 0.8%), inoculation 0.2%, 37 °C, 24 h); *K. rhizophila* ATCC 9341 (nutrient agar, inoculation 0.2%, 37 °C, 24 h); *M. smegmatis* ATCC 607 (Waksman agar (peptone 0.5%, meat extract 0.5%, NaCl 0.3%, glucose 1.0% and agar 0.8%), inoculation 1.0%, 37 °C, 24 h); *Escherichia coli* NIHJ (nutrient agar, inoculation 0.5%, 37 °C, 24 h); *Pseudomonas aeruginosa* IFO 3080 (nutrient agar, inoculation 1.9%, 37 °C, 24 h); *Xanthomonas campestris* KB88 (nutrient agar, inoculation 1.0%, 37 °C, 24 h); *Bacteroides fragilis* ATCC 23745 (GAM medium (GAM broth (Nissui Pharmaceutical, Tokyo, Japan) 5.0% and agar 1.5%), 2.0% inoculation, 37 °C, 24 h); *A. laidlawii* KB174 (Ala medium (PPLO broth (Becton Dickinson) 3.0%, phenol red (5.0 mg ml^{-1}) 0.2%, glucose 0.1%, agar 1.5%, horse serum (Cosmo Bio, Tokyo, Japan) 15.0% and penicillin G) 1.0%, inoculation 20%, 37 °C, 24 h); *Pyricularia oryzae* KF180 (GY agar (glucose 1.0%, yeast extract 0.5% and agar 0.8% adjusted in pH 6.0, inoculation 2.0%, 37 °C, 24 h); *Aspergillus niger* ATCC 9642 (GY agar, inoculation 0.3%, 27 °C, 48 h); *Mucor racemosus* IFO4581 (GY agar, inoculation 0.3%, 27 °C, 48 h); *C. albicans* ATCC 64548 (GY agar, inoculation 0.2%, 27 °C, 24 h); and *Saccharomyces cerevisiae* KF26 (GY agar, inoculation 0.3%, 27 °C, 24 h).

1 Keller, N. P., Turner, G. & Bennett, J. W. Fungal secondary metabolism - from biochemistry to genomics. *Nat. Rev. Microbiol.* **3,** 937–947 (2005).
2 Hoffmeister, D. & Keller, N. P. Natural products of filamentous fungi: enzymes, genes, and their regulation. *Nat. Prod. Rep.* **24,** 393–416 (2007).
3 Sanchez, J. F., Somoza, A. D., Keller, N. P. & Wang, C. C. Advances in *Aspergillus* secondary metabolite research in the post-genomic era. *Nat. Prod. Rep.* **29,** 351–371 (2012).
4 Kawaguchi, M., Nonaka, K., Masuma, R. & Tomoda, H. New method for isolating antibiotic-producing fungi. *J. Antibiot.* **66,** 17–21 (2013).
5 *Dictionary of Natural Products on DVD December 2011* (Chapman & Hall, CRC Press, London, Version 20.2, 2011).
6 Takamatsu, S. *et al.* A novel testosterone 5α-reductase inhibitor, 8′,9′-dehydroascochlorin prooduced by *Verticillium* sp. FO-2787. *Chem. Pharm. Bull.* **42,** 953–956 (1994).
7 Altschul, S. F. *et al.* Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25,** 3389–3402 (1997).
8 von Arx, J. A. *The Genera of Fungi Sporulating in Pure Culture.* 3rd edn 273 (Verlag J Cramer, Vaduz, 1981).
9 Ivanova, V., Backor, M., Dahse, H. M. & Graefe, U. Molecular structural studies of lichen substances with antimicrobial, antiproliferative, and cytotoxic effects from *Parmelia subrudecta*. *Prep. Biochem. Biotechnol.* **40,** 377–388 (2010).
10 Sanchez, J. F. *et al.* Molecular genetic analysis of the orsellinic acid/F9775 gene cluster of *Aspergillus nidulans*. *Mol. Biosyst.* **6,** 587–593 (2010).
11 Singh, S. B. *et al.* Chemistry and Biology of Cylindrols: Novel Inhibitors of Ras Farnesyl-Protein Transferase from *Cylindrocarpon lucidum*. *J. Org. Chem.* **61,** 7727–7737 (1996).
12 Gutiérrez, M., Theoduloz, C., Rodríguez, J., Lolas, M. & Schmeda-Hirschmann, G. Bioactive metabolites from the fungus *Nectria galligena*, the main apple canker agent in Chile. *J. Agric. Food Chem.* **53,** 7701–7708 (2005).
13 Knothe, G. & Kenar, J. Determination of the fatty acid profile by ^{1}H-NMR spectroscopy. *Eur. J. Lipid Sci.* **106,** 88–96 (2004).
14 Mori, K. & Fujioka, T. Synthesis of (±)-ascochlorin, (±)-ascofuranone and LL-Z1272. *Tetrahedron* **40,** 2711–2720 (1984).
15 Seephonkai, P., Isaka, M. & Kittakoop, P. A novel ascochlorin glycoside from the insect pathogenic fungus *Verticillium hemipterigenum* BCC 2370. *J. Antibiot.* **57,** 10–16 (2004).
16 Chaverri, P., Salgado, C., Hirooka, Y., Rossman, A. Y. & Samuels, G. J. Delimitation of *Neonectria* and *Cylindrocarpon* (*Nectriaceae*, *Hypocreales*, *Ascomycota*) and related genera with Cylindrocarpon-like anamorphs. *Stud. Mycol.* **68,** 57–78 (2011).
17 Seifert, K., Morgan-Jones, G., Gams, W. & Kendrick, B. *The Genera of Hyphomycetes. CBS Biodiversity Series 9* (CBS-KNAW Fungal Biodiversity Centre, Utrecht, 2011).
18 Horsburgh, T., Wood, P. & Brent, L. Suppression of *in vitro* lymphocyte reactivity by cyclosporin A: existence of a population of drug-resistant cytotoxic lymphocytes. *Nature* **286,** 609–611 (1980).
19 Evans, G. & White, N. H. Radicicolin and radicicol, two new antibiotics produced by *Cylindrocarpon radicicola*. *Trans. Br. Mycol. Soc.* **49,** 563–576(1966).
20 Hunter, R. & Mellows, G. Detection of deuteride shifts in the biosynthesis of the fungal triprenylphenol, ascochlorin, by ^{13}C nuclear magnetic resonance spectroscopy following incorporation of [3-^{13}C, 4-$^2H_2$]-mevalonic acid. *Tetrahedron Lett.* **50,** 5051–5054 (1978).
21 Vilella, D. *et al.* Inhibitors of farnesylation of Ras from a microbial natural products screening program. *J. Ind. Microbiol. Biotechnol.* **25,** 315–327 (2000).
22 Atlas, R. M. *Handbook of Microbiological Media.* 4th edn (CRC Press, Boca Raton, 2010).
23 Jacobson, E., Granville, W. C. & Foss, C. E. *Color Harmony Manual.* 4th edn (Container Corporation of America, Chicago, IL, USA, 1958).
24 White, T. J., Bruns, T., Lee, S. & Taylor, J. W. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M. A., Gelfand, R. H., Sninsky, J. J. & White, T. J. (eds). *PCR Protocols: A Guide to Methods and Applications.* 315–332 (Academic Press, New York, 1990).
25 Iwatsuki, M. *et al.* Lariatins, novel anti-mycobacterial peptides with a lasso structure, produced by *Rhodococcus jostii* K01-B0171. *J. Antibiot.* **60,** 357–363 (2007).