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FULL PAPER

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Taxonomy and secondary metabolites of *Pseudobotrytis* sp. FKA-25

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Abstract Fungal strain FKA-25, isolated from forest soil collected on Yakushima Island, Kagoshima Prefecture, Japan, was assigned to genus *Pseudobotrytis* based on its morphological characteristics. Conidiophores were erect, slightly swollen at the end of the tip, and gave rise to umbellate conidiogenous cells that were in an expanded denticulate portion at the end and formed ellipsoidal to clavate conidia in sympodial succession. Identification as species *P. terrestris* was made on the basis of the character of 1-septate conidium. Although no secondary metabolites have been reported from the genus *Pseudobotrytis*, four secondary metabolite compounds (designated A to D) were isolated from the culture broth of strain FKA-25. Compounds B to D have been reported previously as FK-17-p2a, lunatinin, and 3,4-dihydro-3,4,8-trihydroxy-1(2*H*)-naphthalenone, respectively. Compound A was designated sespendole and possessed a novel indole-sesquiterpene skeleton.

Key words Drug discovery · Indole-sesquiterpene · *Pseudobotrytis terrestris* FKA-25 · Secondary metabolite

Introduction

Among fungi, the genera *Penicilluim*, *Aspergillus*, *Trichoderma*, *Fusarium*, *Gliocladium*, and *Acremonium* have appeared in a considerable number of reports as producers of not only antimicrobial agents but also a variety of bioactive compounds including enzyme inhibitors, receptor antagonists/agonists, and immunomodulators (Turner 1971; Turner and Aldrige 1983; Tomoda 2002). Over the past two decades, fungi have attracted attention as an important resource for the discovery of new bioactive compounds. It is well known that fungi are the largest group among microorganisms; there are estimated 65 000 species of teleomorphic fungi and about 16 000 species of anamorphic fungi (Kirk et al. 2001). We are attempting to develop fungal resources for drug discovery by characterizing previously unknown fungi or even known fungi that have been rarely reported to produce secondary metabolites.

Fungal strain FKA-25 was isolated from a soil sample collected at Yakushima Island, Kagoshima Prefecture. In this article, we show that FKA-25 belongs to the genus *Pseudobotrytis* (Domsh et al. 1993) based on taxonomic characteristics. There have been some reports on this genus, but no reports on the secondary products. The secondary metabolites produced by strain FKA-25 are also described.

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Materials and methods

Strains

Fungal strain FKA-25 was isolated from forest soil collected on Yakushima Island, Kagoshima Prefecture, Japan. As authentic strains, *Pseudobotrytis terrestris* ATCC 44871, ATCC 10095, and *Pseudobotrytis bisbyi* ATCC 16248 were obtained from the American Type Culture Collection, Manassa, VA, USA, and *Pseudobotrytis terrestris* NBRC 7064 and NBRC 9213 were obtained from the Biological Resource Center, Biotechnology Center, National Institute of Technology and Evaluation, Chiba, Japan. These strains were maintained on YpSs slants (15 g soluble starch, 4 g yeast extract, 1 g K_2HPO_4, 0.5 g $MgSO_4{\cdot}7H_2O$, and 20 g agar in 1 l distilled water) or modified LCA slants (1 g glycerol, 0.8 g KH_2PO_4, 0.2 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.2 g KCl, 2 g $NaNO_3$, 0.2 g yeast extract, and 15 g agar in 1 l distilled water).

Media

For morphological characterization, potato dextrose agar (PDA; Difco, Sparks, MD, USA), cornmeal agar (CMA; Difco), and modified LCA slants were used.

For production of strain FKA-25 metabolite compounds identified A to D, seed medium (20 g glucose, 2 g yeast extract, 0.5 g $MgSO_4 \cdot 7H_2O$, 5 g Polypepton (Nihon Pharmaceutical, Tokyo, Japan), 1 g KH_2PO_4, and 1 g agar in 1 l tap water) and production medium (20 g sucrose, 10 g glucose, 5 g corn steep powder, 5 g meat extract, 1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.01 g $MnCl_2 \cdot 4H_2O$, 0.01 g $ZnSO_4 \cdot 7H_2O$, 0.01 g $CuSO_4 \cdot 5H_2O$, 0.01 g $CoCl_2 \cdot 2H_2O$, 3 g $CaCO_3$, and 1 g agar in 1 l tap water) were used.

Morphological observations

Each fungal strain was inoculated on the center of 90-mm glass dishes containing each of the three types of agar medium and incubated at 25°C for 7–14 days under darkness. The color of the colonies was described by hues according to the Color Harmony Manual (Taylor et al. 1958). Morphological observations were carried out under a light microscope (Vanox-S AH-2; Olympus, Tokyo, Japan) and scanning electron microscope (SEM; JSM 5600, JEOL, Tokyo, Japan). For SEM observation, 5-mm-diameter mycelial disks of the colonies were cut from the LCA plates, fixed with the vapor from crystalline OsO_4 in a glass-stoppered flask for 1 h at room temperature, coated with gold using a JFC-1200 Fine Coater (JEOL), and observed under the SEM at 15 kV.

Production, isolation, and purification of compounds A to D

Strain FKA-25 grown on an LCA slant was inoculated into a 500-ml Erlenmeyer flask containing 100 ml seed medium, which was shaken at 210 rpm on a rotary shaker for 3 days at 27°C. The seed culture (200 ml) was transferred into a 30-l jar fermentor containing 20 l production medium. The fermentation was carried out at 27°C for 5 days under agitation of 250 rpm and aeration of 10 l/min.

The 5-day-old whole culture was used for purification of compounds A to D. Silica gel 60 (70–230 or 230–400 μm; Merck, Darmadt, Germany) was used for column chromatography. High pressure liquid chromatography (HPLC) (Hitachi HPLC system, Tokyo, Japan) was carried out for final purification of the compounds.

Spectroscopic studies of compounds

Fast atom bombardment (FAB) mass spectrometry was conducted on a JMS-AX505H spectrometer (JEOL). Ultraviolet (UV) and infrared (IR) spectra were measured with a DU640 spectrophotometer (Beckman, Fullerton, CA, USA) and a FT-210 Fourier transform infrared spectrometer (Horiba, Tokyo, Japan), respectively. Nuclear magnetic resonance (NMR) spectra were obtained with an EX-270 spectrometer (JEOL) and an XL-400 spectrometer (Varian, Palo Alto, CA, USA).

Analysis of *Pseudobotrytis* spp. metabolites by LC/UV

For analysis of secondary metabolites produced by *Pseudobotrytis* spp., each strain on an LCA slant was inoculated into a test tube containing 10 ml seed medium. The tube was shaken at 27°C for 3 days. The seed culture (1 ml) was transferred to a 500-ml Erlenmeyer flask containing 100 ml production medium. Fermentation was carried out on a rotary shaker (210 rpm) at 27°C. The 5-day-old culture (1 ml) was extracted with an equal volume of EtOH. After centrifugation, the supernatant (1 ml) was concentrated to remove EtOH. Then, the water suspension was extracted with ethyl acetate (0.5 ml). The ethyl acetate layer (0.4 ml) was concentrated to dryness and the remaining material was dissolved in methanol (0.1 ml) to analyze the metabolites by liquid chromatography with UV photodiode array detection (LC/UV) (HP 1100 system; Hewlett Packard, Palo Alto, CA, USA) using a Symmetry C18/3.5 μm column (i.d. 2.1 × 150 mm; Waters, Milford, MA, USA) with a mobile phase on a 20-min linear gradient from 5% CH_3CN/0.05% H_3PO_4 to 100% CH_3CN/0.05% H_3PO_4 with a flow rate of 0.2 ml/min. Peaks representing compounds A, B, C, and D were eluted with retention times of 16.7, 9.7, 10.8, and 4.9 min, respectively (see Fig. 5).

Results

Morphological characteristics of strain FKA-25

The appearance of colonies of strain FKA-25 incubated at 25°C for 14 days on PDA, CMA, and LCA is shown in Fig. 1. Colonies on PDA (Fig. 1A) were 67–69 mm in diameter, velvety to farinaceous, dark olive (1.5 nl) to beige-gray (3 ih) in color with abundant sporulation; the reverse side was dark brown (3 pn) to clove-brown (3 ni). Colonies on CMA (data not shown) were 42–44 mm in diameter, thin, hyaline, and mustard-brown (2 pl) in some places with poor sporulation. Colonies on LCA (data not shown) were 36–40 mm in diameter, thin, hyaline, and olive (1 pl) to mustard-brown (2 pl) in some places with poor sporulation.

After 7 and 14 days on LCA, morphological properties were observed under a light microscope and an SEM. Conidiophores were erect, consisting of unbranched straight stipes with 5–7 septates, brown in the lower part, almost hyaline at the tip, and 180–390 × 4.5–6.0 μm (Fig. 1B), and were supported by the basal rhizomorphoid cells (Fig. 1C). Chlamydospores were globose to subglobose (5.5–8.5 μm) or obovate (8.5–12 × 7–8 μm), pale brown to dark brown (Fig. 1D). Conidiogenous cells arose from the smaller expanded apex of the conidiophores, and were 6–14 umbellate, a few tapering toward the tip (Fig. 1E). Upon maturing, conidiogenous cells expanded to 4–5.5 μm in diameter and formed a number of denticulate protuberances at the end (Fig. 1F). Conidia were produced in sympodial succession, ellipsoidal to clavate with apiculate base, 1-septate, pale brown in color, 9–11 × 4.3–5.2 μm (Fig. 1E,F). Conidial septa were found to expand under SEM observation (Fig. 1F). Based on these characteristics, the strain was

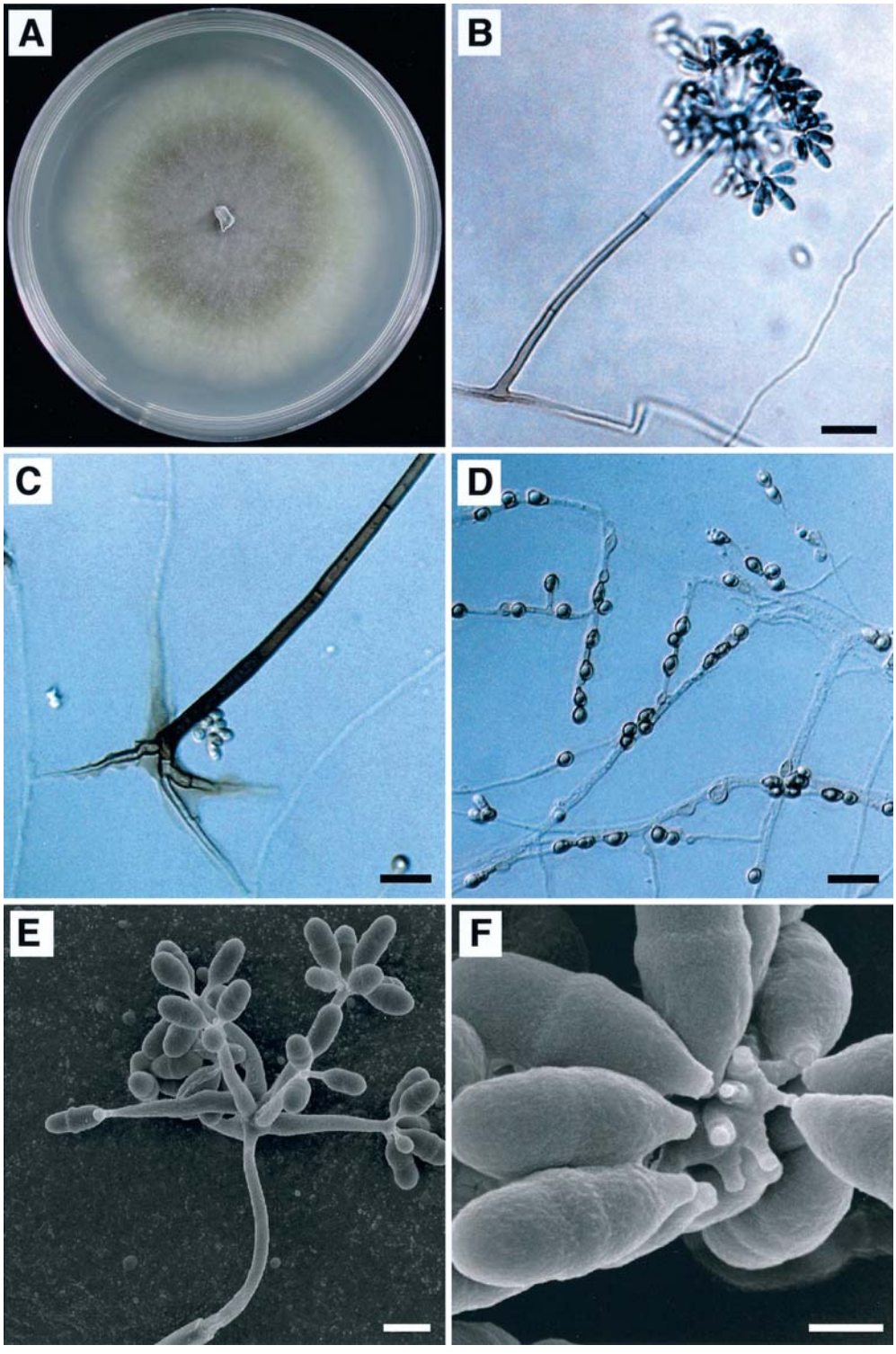


Fig. 1. Morphological characteristics of fungal strain FKA-25. **A** Colony on PDA at 25°C for 14 days. **B** Conidiophore and umbellate conidiogenous cells strained with lactophenol cotton blue. **C** Basal rhizomorphoid cell. **D** Chlamydospores. **E** Umbellate conidiogenous cells under SEM. **F** Denticulate protuberance in an expanded portion and conidia in sympodial succession (SEM). *Bars* **B–D** 20 μm; **E** 5 μm; **F** 2 μm

identified as belonging to the genus *Pseudobotrytis* Krezem. and Badura (Domsh et al. 1993). Furthermore, *P. terrestris* (Timonin) Subramanian has conidial septa (Fig. 2A,B), whereas *P. bisbyi* Timonin has non-septate conidia (Fig. 2C) of similar size to *P. terrestris*. Strain FKA-25 also had conidial septa, and was identified as *P. terrestris*.

Purification and structure of compounds A to D

The purification of compounds A to D from the culture of strain FKA-25 is summarized in Fig. 3. The 5-day-old whole culture was extracted with 20 l acetone. After centrifugation of the mixture, the supernatant was concentrated to remove

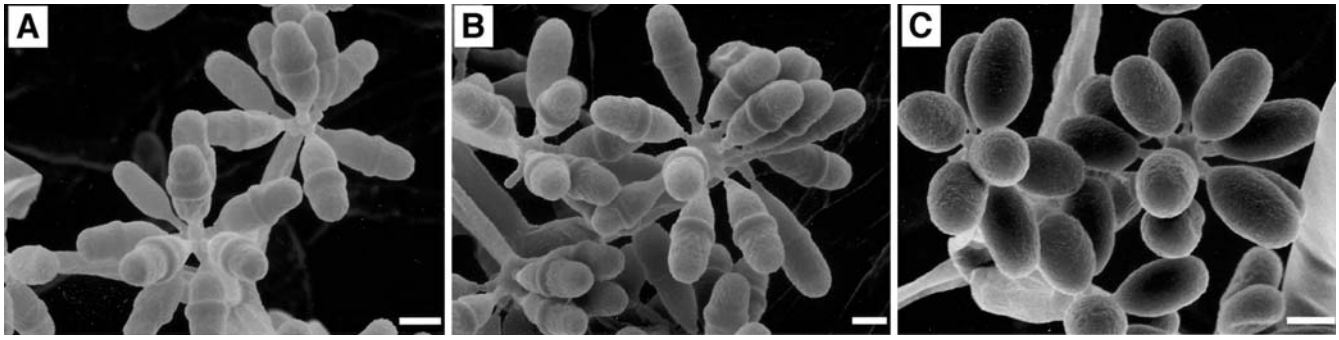
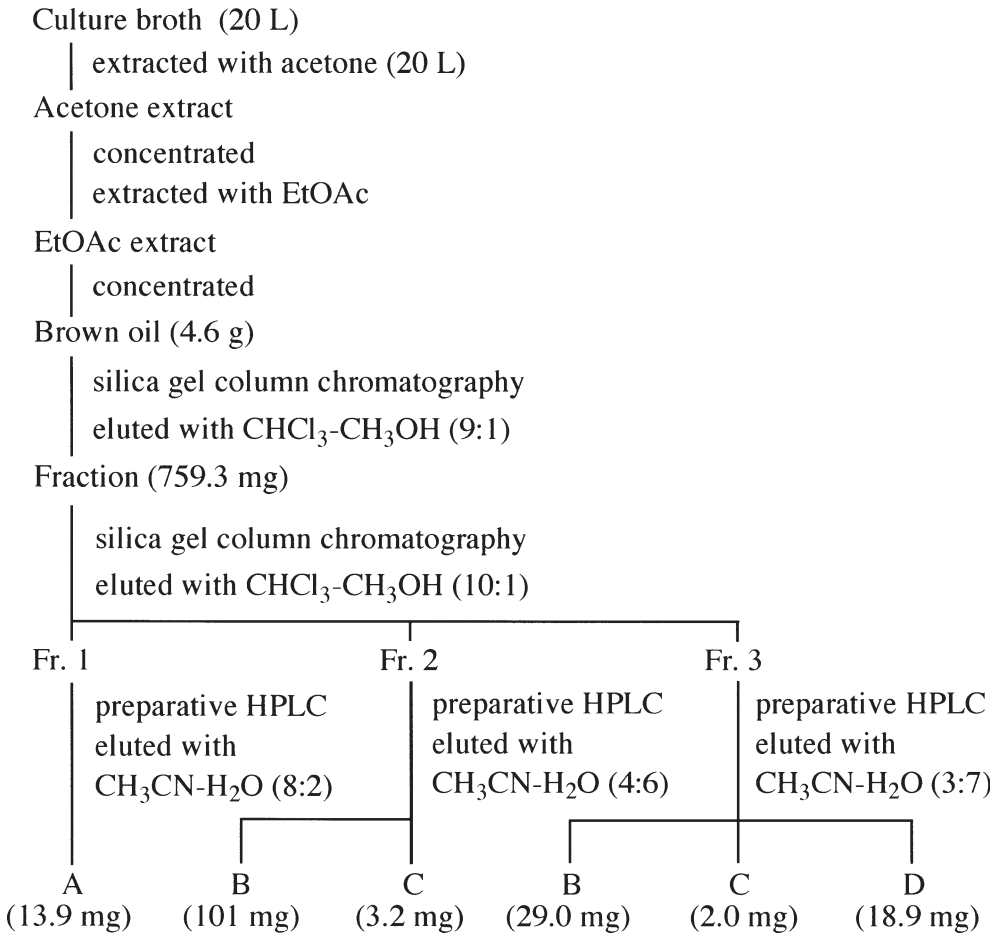


Fig. 2. Conidiogenous cells and conidia of *Pseudobotrytis* spp. **A** *Pseudobotrytis terrestris* ATCC 44871. **B** *Pseudobotrytis terrestris* NBRC 7064. **C** *Pseudobotrytis bisbyi* ATCC 16248. *Bars* 4 μm

Fig. 3. Purification procedure of compounds *A* to *D*



acetone and the aqueous suspension was extracted with 201 ethyl acetate. The extract was concentrated in vacuo to dryness to yield a brown oil residue (4.6 g). The oil was purified with a silica gel column (i.d. 2.5 × 16 cm, 70–230 μm) by stepwise additions of chloroform : methanol solutions (1 l each 100 : 0, 50 : 1, 9 : 1, 1 : 1, and 0 : 100). Fractions (759 mg) eluted in the chloroform : methanol (9 : 1) were applied to a second silica gel column (i.d. 2.0 × 20 cm, 230–400 μm) by stepwise additions of chloroform : methanol solutions (200 ml each 100 : 0, 50 : 1, 10 : 1, 5 : 1, and 0 : 100). Compounds A to D were eluted in the 10 fractions (20 ml × 10) of the 10 : 1 chloroform : methanol solution. Compound A (132 mg, yellow powder) was obtained from the 1st to 3rd fractions (hereafter, Fr. 1), compounds B and C (316 mg, brown powder) were recovered from the 4th to 6th fractions (hereafter, Fr. 2), and compounds B to D (171 mg, brown powder) were recovered from the 7th to 10th fractions (hereafter, Fr. 3). Fr. 1, 2, and 3 were purified by reversephase HPLC using a Pegasil ODS column (i.d. 20 × 250 mm; Senshu, Tokyo, Japan) with a flow rate of 8 ml/min and the following mobile phases: 80% aq. CH_3CN (for Fr. 1), 40% aq. CH_3CN (for Fr. 2), or 30% aq. CH_3CN (for Fr.

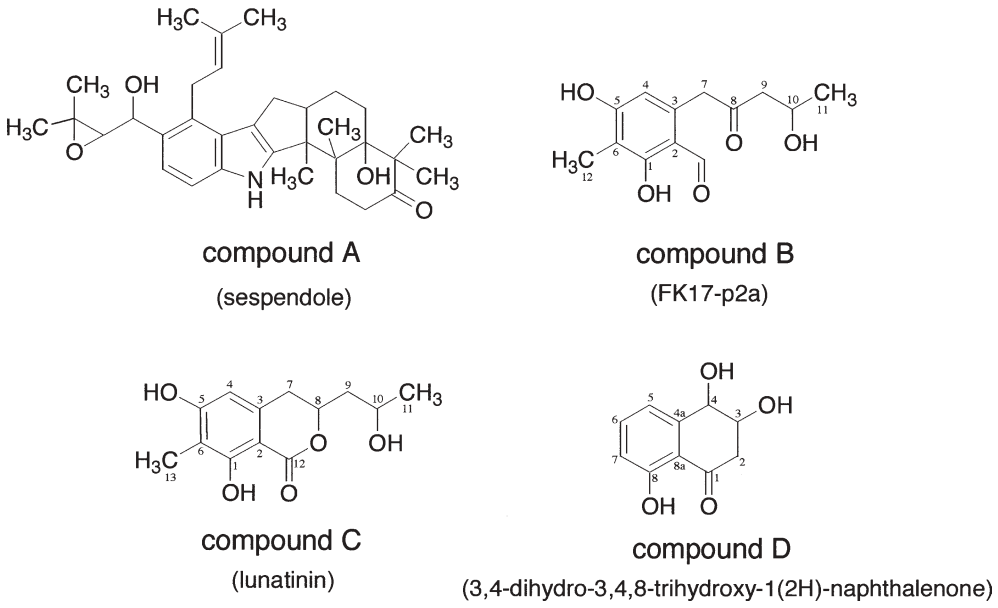


Fig. 4. Structure of compounds A to D produced by *Pseudobotrytis terrestris* FKA-25. Number in the structures of compounds B to D correspond to the carbon numbers in Table 2

Table 1. Physicochemical properties of compounds B to D

	Compound B	Compound C	Compound D
Apperance	Orange powder	Ocher powder	White amorphous
Molecular weight	252	250	194
Molecular formula	$C_{13}H_{16}O_5$	$C_{13}H_{14}O_6$	$C_{10}H_{10}O_4$
Pos. FAB-MS (m/z)	253 $(M + H)^+$	251 $(M + H)^+$	195 $(M + H)^+$
UV λ max^{MeOH}nm	211, 242sh, 296, 340sh	240sh, 248, 282sh, 334	224, 260, 322
$[\alpha]D^{23}$(MeOH)	−8.0 (*c*, 0.1)	+22.0 (*c*, 0.1)	−32.0 (*c*, 0.1)
IR $\nu_{max}^{KBr}cm^{-1}$	1155, 1116, 1440, 1627, 1672, 3131, 3363	1122, 1255, 1311, 1616, 3166, 3388	1282, 1589, 1673, 3226, 3417

3). Detection was carried out at 220 nm. The fraction of a peak representing compound A was eluted with a retention time of 7.2 min from Fr. 1, the fractions containing compounds B and C were eluted with retention times of 13.9 and 19.1 min from Fr. 2, respectively, and the fractions containing compounds B, C, and D were eluted with retention times of 14.3, 19.6, and 9.0 min. After evaporation of the fractions of each compound combined, compounds A (13.9 mg), B (130 mg), C (5.2 mg), and D (18.9 mg) were obtained as white amorphous, orange powder, ocher powder, and white amorphous, respectively.

The structures of the compounds are shown in Fig. 4. Compounds B, C, and D were known, but compound A was newly discovered and named sespendole. Compound A (sespendole) possessed a novel indole-sesquiterpene skeleton structure. The structure elucidation and biological activity will be published elsewhere. The spectral and physical data for compounds B, C, and D have not been reported in detail to date and are summarized in Tables 1 and 2. Furthermore, ^1H and ^{13}C signals in NMR spectra (Table 2) were clearly assigned to each position in the structures as numbered in Fig. 4. Compound B was identified as FK17-P2a, and was previously reported as an UV ray-absorbing substance produced by *Aspergillus versicolor* (Arai and Sano 1994). Compound C was identified as lunatinin (Nukina et al. 1978), an intermediate of lunatoic acid A produced by *Cochliobolus lunata* (Marumo et al. 1982). Lunatoic acid A was originally reported as an aversion factor, which inhibits mycelial growth of different strains in the same fungal species (Nukina 1987). Compound D was identified as 3,4-dihydro-3,4,8-trihydroxy-1(2*H*)-naphthalenone, a phytotoxic substance produced by *Pyricularia oryzae* (Iwasaki et al. 1972).

Characterization of *Pseudobotrytis* spp. metabolites

Ethyl acetate extracts from the 5-day-old culture of strain FKA-25 and five *Pseudobotrytis* authentic strains were analyzed by LC/UV to compare the metabolites. As shown in Fig. 5, compounds A (retention time, 16.7 min), B (9.7 min), C (10.8 min), and D (4.9 min) were observed in the culture broth of FKA-25. The peak corresponding to compound D was detected in almost all *Pseudobotrytis* spp. (except *P. terrestris* ATCC 10095), and multiple peaks with retention times of 6.0 min (UV_{max} 225, 275 nm, analyzed by LC/UV), 7.0 min (UV_{max} 225, 260, 320 nm), 7.5 min (UV_{max} 205 nm), and 8.0 min (UV_{max} 205 nm) were observed in almost all the strains. Although compounds correspounding to the multiple peaks were not analyzed in this study, they have common retention times and UV absorptions. On the other hand, compounds A, B, and C were only observed in the

Table 2. ^{1}H and ^{13}C NMR chemical shifts of compounds B to D

No.	Compound B (ppm)		No.	Compound C (ppm)		No.	Compound D (ppm)	
	^{13}C Chemical Shifts (Mult.[a])	^{1}H Chemical Shifts (Mult.[a])		^{13}C Chemical Shifts (Mult.[a])	^{1}H Chemical Shifts (Mult.[a])		^{13}C Chemical Shifts (Mult.[a])	^{1}H Chemical Shifts (Mult.[a])
1	165.0 (s)		1	167.5		1	199.3 (s)	
2	110.9 (s)		2	99.5		2	42.9 (t)	3.26 (dd)
3	139.4 (s)		3	137.7 (s)				2.76 (dd)
4	111.8 (d)	6.46 (s)	4	106.3 (d)	6.33 (s)	3	72.0 (d)	4.44 (m)
5	163.7 (s)		5	164.3 (s)		4	67.4 (d)	6.33 (d)
6	113.9 (s)		6	111.1 (d)		4a	134.3 (s)	
7	47.9 (t)	4.25 (s)	7	102.5 (d)	6.25 (s)	5	130.3 (d)	7.15 (t)
8	207.5 (s)		8	161.7 (s)		6	118.4 (d)	7.36 (d)
9	52.2 (t)	2.77 (m)	9	43.8 (t)	2.42 (t)	7	122.3 (d)	6.98 (d)
10	65.1 (d)	4.31 (q)	10	65.5 (d)	4.00 (m)	8	158.6 (s)	
11	24.2 (q)	1.26 (d)	11	23.6 (q)	1.10 (d)	8a	129.4 (s)	
12	195.2 (d)	9.90 (s)	12	155.5 (s)				
13	7.5 (q)	2.12 (s)	13	8.0 (q)	1.95 (s)			
1-*COH*		12.82 (s)	1-*COH*		11.32 (s)			

Compounds B and C measured in acetone-d_6; compound D measured in methanol-d_4
[a] Multiplicity: s, singlet; d, doublet; t, triplet; q, quartet; dd, double doublet; m, multiplet

culture of strain FKA-25 and were not detected in the culture of the other *Pseudobotrytis* spp.

Discussion

Strain FKA-25 was identified as a member of genus *Pseudobotrytis* (Domsh et al. 1993) based on morphological characteristics (see Fig. 1). There have been several reports on the isolation of *P. terrestris* in Europe, North America, New Zealand, Jamaica, Panama, and Japan (Domsh et al. 1993). The species was isolated from cultivated soil (Robison 1970), forest soil (Hammill 1970) and arable soil, and decaying stems and plant leaves (Badurova and Badura 1967; Ellis 1971; Matsushima 1975). These reports indicate that *P. terrestris* is distributed widely in nature. Strain FKA-25 was isolated from soil collected in evergreen-leaved forests populated by mainly *Litsea acuminata* (Bl.) Kurata and *Machilus japonica* Seib. and Zucc.

Despite the presumed common distribution of *Pseudobotrytis* strains, only two species, *P. terrestris* and *P. bisbyi* (Matsushima 1975; Timonin 1961), have been reported; these two species are differentiated by the presence of 1-septate (Fig. 2A,B) or aseptate conidia (Fig. 2C), respectively (Domsh et al. 1993). Because FKA-25 has umbellate denticulate conidiogenous cells producing 1-septal conidia (Fig. 1E, F), it was considered to belong to *P. terrestris*.

Our research group has focused on fungal strains as an important resource for the discovery of useful bioactive compounds (Tomoda 2002). Interestingly, there have been no reports on secondary metabolites from *Pseudobotrytis* strains. Therefore, the culture of strain FKA-25 was analyzed, and four compounds were isolated from acetone extracts of the culture (see Fig. 3). Elucidation of their structures from the spectral data (see Tables 1, 2) showed that three compounds (Fig. 4), i.e., B (FK17-P2a) (Arai and Sano 1994), C (lunatinin) (Nukina et al. 1978), and D (3,4-dihydro-3,4,8-trihydroxy-1(2*H*)-naphthalenone) (Iwasaki et al. 1972), were previously known to be produced by different genera (*Aspergillus*, *Cochliobolus*, and *Pyricularia*, respectively), but compound A (sespendole) was found to be a new compound having inhibitory activity against lipid droplet accumulation in macrophages (a cellular model of screening for antiatherosclerotic agents) (Namatame et al. 1999). Sespendole is the first compound with an indole-sesquiterpene skeleton, although a number of indole-diterpenes have been reported to be produced by various fungi: paspaline and paspalicine produced by *Claviceps paspali* (Springer and Clardy 1980), paxilline by *Peniciilium paxilli* (Springer et al. 1974), janthitrems by *Penicillium janthinellum* (Gallagher et al. 1980), and terpendoles by *Albophoma yamanashiensis* (Huang et al. 1995). Thus, we have shown that a *Pseudobotrytis* strain produces a compound with a unique chemical skeleton and biological activity. The details of the structure and biological activity of sespendole will be published elsewhere.

Furthermore, ethyl acetate extracts of cultures from six *Pseudobotrytis* strains were analyzed by LC/UV to determine the relationship between the species and their metabolites (Fig. 5). Okuda et al. (1982, 2000) proposed that some species of *Trichoderma* and *Gliocladium* are differentiated by specific metabolites, as certain *Trichoderma* species produce isonitrile antibiotics and certain *Gliocladium* species produce TMC-151, 154, and 171 compounds. In this studies, *Pseudobotrytis* species produced multiple peaks with common retention times (see Fig. 5) and UV absorption, suggesting that they might be common metabolites of *Pseudobotrytis* spp. However, under the conditions used in this study, compounds A to C were detected only in the culture broth of *P. terrestris* FKA-25. To elucidate this possibility in *Pseudobotrytis* spp., more detailed investigation of culture condition is required.

A number of fungi have been reported, but in most cases their metabolites have not been studied in detail. In this

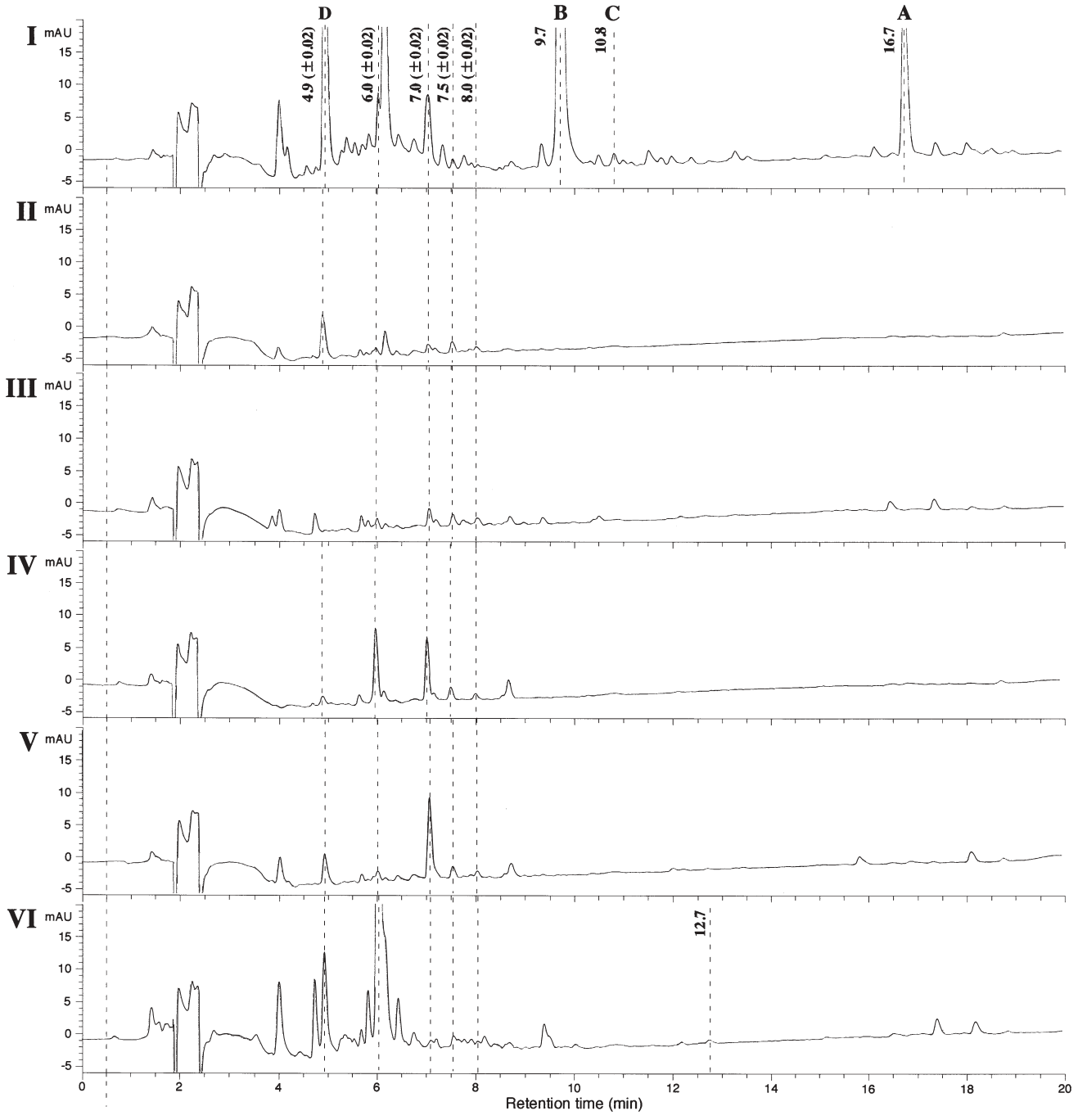


Fig. 5. Liquid chromatography with UV photodiode array detection (LC/UV) chromatograms at 220 nm of *Pseudobotrytis* spp. metabolites. **I** *Pseudobotrytis terrestris* FKA-25. **II** *Pseudobotrytis terrestris* ATCC 44871. **III** *Pseudobotrytis terrestris* ATCC 10095. **IV** *Pseudobotrytis terrestris* NBRC 7064. **V** *Pseudobotrytis terrestris* NBRC 9213. **VI** *Pseudobotrytis bisbyi* ATCC 16248. Peaks *A*, *B*, *C*, and *D* correspond to compounds A, B, C, and D

article, we presented known fungi whose metabolites had not been reported but have potential as useful resources for the discovery of new bioactive compounds.

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