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Phytochemistry 65 (2004) 469–473
PHYTOCHEMISTRY
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Cyclic azaphilones daldinins E and F from the ascomycete fungus *Hypoxylon fuscum* (Xylariaceae)

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Received 6 August 2003; received in revised form 24 September 2003

Abstract

Phytochemical examination of the methanolic extract of fruit bodies of the Xylariaceous ascomycete fungus *Hypoxylon fuscum* led to the isolation of two azaphilone derivatives named daldinins E and F together with two known compounds daldinin C and 4,5,4′,5′-tetrahydroxy-1:1′-binaphthyl using a combination of reversed phase HPLC and high performance gel permeation chromatography. Their structures were determined by 2D NMR, MS, IR, UV, and CD spectroscopy. Their antioxidative activities were also estimated by an indication of a 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging effect. The chemosystematics of *Hypoxylon* is discussed.

Keywords: *Daldinin*; *Hypoxylon*; Xylariaceae; Fungus; Azaphilone; Chemosystematics

1. Introduction

Xylariaceous fungi, and in particular the genera *Daldinia* Ces. & De Not. and *Hypoxylon* Bull., have been shown to contain manifold secondary metabolites, and their production appears to be specific for certain species, species groups, or genera (Whalley and Edwards, 1995). Among these metabolites, azaphilones of the mitorubrin and daldinin types, as well as binaphthalenes and benzophenones (Hashimoto et al., 1994a,b; Hashimoto and Asakawa, 1998; Stadler et al., 2001a,c) in the fruitbodies (i.e. stromata) of these fungi, are responsible for the taxonomically significant pigment reaction in 10% KOH (Ju and Rogers, 1996; Ju et al., 1997) of their ectostroma (i.e. the outermost part of their stromata that also contains the ascigenous structures). In addition, several uncolored chemical matters such as cytochalasins and concentricols (Buchanan et al., 1995, 1996a,b; Hashimoto and Asakawa, 1998; Stadler et al. 2001b; Quang et al. 2002a,b) are also characteristic of certain species. As shown for *Daldinia concentrica*, the minor metabolites of these fungi also include highly oxidized binaphthalene derivatives and other aromatic components, as well as various types of small polyketides (Quang et al., 2002b). Studies by Mühlbauer et al. (2002) on *Hypoxylon fuscum* (Pers.:Fr.) Fr. and *H. fuscopurpureum* (Schwein.) also revealed the presence of other specific and thus taxonomically significant metabolites, e. g. daldinal A and daldinin C. Recently, stromata of *H. fuscum* were collected in large quantities, allowing for an intensified study of its chemical constituents. The outcome of these efforts is the subject of the current paper.

2. Results and discussion

The methanolic extract prepared from freshly collected fruit bodies of *H. fuscum* was subjected repeatedly to reversed-phase HPLC, SiO_2, and Sephadex LH-20 column chromatography, followed by high performance gel permeation chromatography (HPGPC) on Lichrogel PS1 (as described in the Experimental) to yield compounds **1–4**. The latter technique was

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doi:10.1016/j.phytochem.2003.09.022

employed because the two-component mixtures appeared hardly separable using conventional HPLC and other aforementioned chromatographic methods. HPGPC had proven successful with other mixtures of isomeric fungal metabolites in the past (Stadler et al., 1993). The new *spiro*-azaphilones obtained in the course of the current study were named daldinins E (**1**) and F (**2**) as addition to the previously described daldinins A-C from *Daldinia* spp. (Hashimoto et al., 1994b). There has been a report on a compound named daldinin D from *Penicillium thymicola* by Ariza et al. (2001). Daldinin D is the only compound of this type, which has so far been found in fungi not belonging to the Xylariaceae.

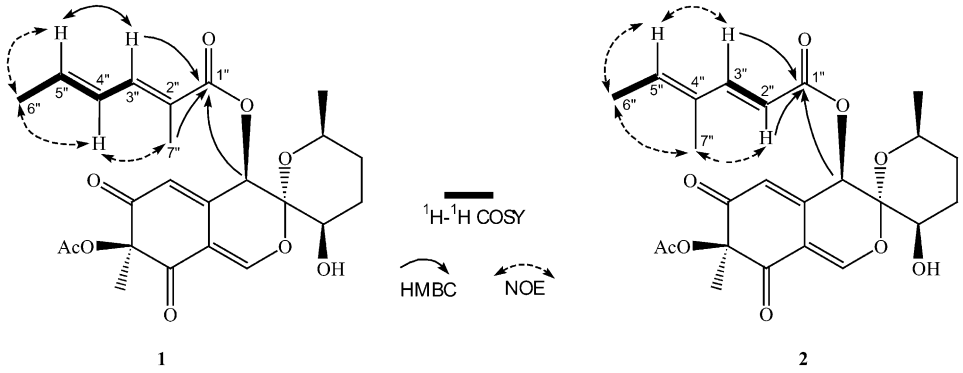
Daldinin E (**1**) was obtained as an oil with the molecular formula of $C_{24}H_{28}O_9$ as determined by HR-FABMS. Its IR and UV spectra showed absorption bands for an ester (1714 cm^{-1}), a conjugated ketone (1675 cm^{-1}, 268 nm), and a conjugated double bond (1628 cm^{-1}, 219 nm). The ^1H NMR spectral data of **1** (Table 1) showed the presence of five olefinic protons, four methyls, and one acetyl group. The ^{13}C NMR spectral data of **1** (Table 2) displayed 24 carbon signals including two ketones, two esters, eight olefinic carbons, two methylenes, and three methines. A comparison of its ^1H–^1H COSY, NOESY, and HMBC spectra with those of daldinin C (**3**), which was originally determined by X-ray crystallographic analysis (Hashimoto et al., 1994b; Hashimoto and Asakawa, 1998) revealed that compound **1** possessed an azaphilone structure highly similar to daldinin C (Tables 1 and 2), only differing from the latter compound in its substitution group at C-4. The ^1H–^1H COSY spectrum of **1** revealed correlations

Table 1
^1H NMR spectral data for **1–3** (600 MHz, $CDCl_3$)

H	1	2	3
1	7.70 *d* 1.4	7.68 *d* 1.4	7.69 *d* 1.4
4	5.79 *s*	5.80 *s*	5.80 *s*
5	6.11 *d* 1.4	6.11 *d* 1.4	6.13 *d* 1.4
3′	3.75 *t* 3.0	3.76 *t* 3.0	3.76 *t* 2.8
4′	2.14 *m*	2.15 *m*	2.13 *br t* 14.3
	1.88 *m*	1.94 *m*	1.93 *ddd* 3.0, 6.6, 14.3
5′	1.80 *m*	1.81 *m*	1.80 *m*
	1.50 *m*	1.50 *m*	1.50 *m*
6′	3.82 *ddd* 2.5, 6.3, 11.8	3.81 *ddd* 2.5, 6.3, 11.8	3.83 *ddd* 2.5, 6.3, 11.8
6′-Me	1.13 *d* 6.3	1.13 *d* 6.3	1.13 *d* 6.3
7-Me	1.54 *s*	1.54 *s*	1.53 *s*
7-CH_3CO	2.17 *s*	2.18 *s*	2.17 *s*
2″		5.75 *d* 15.7	
3″	7.13 *d* 11.3	7.37 *d* 15.7	6.88 *dq* 1.1, 7.1
4″	6.33 *ddd* 1.7, 11.3, 14.8		1.79 *d* 7.1
5″	6.18 *qd* 7.1, 14.8	6.07 *q* 7.1	1.80 *s*
6″	1.88 *d* 7.1	1.83 *d* 7.1	
7″	1.88 *s*	1.75 *s*	
3′-OH			3.24 *d* 5.2

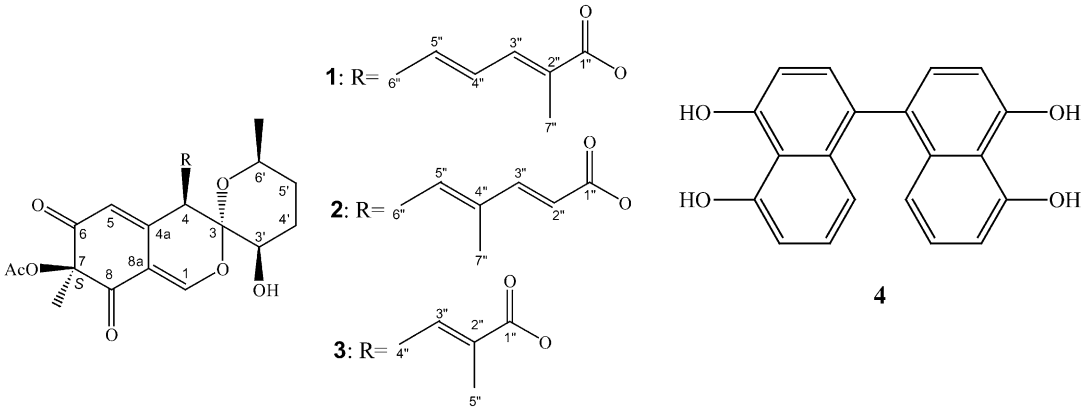
Table 2
^{13}C NMR spectral data for **1–3** (150 MHz, $CDCl_3$)

H	1	2	3
1	154.7	154.7	154.6
3	102.5	102.5	102.5
4	67.2	66.8	67.0
5	121.9	121.9	121.9
6	194.1	194.0	194.0
7	84.9	85.1	85.0
8	192.3	192.3	192.2
3′	62.0	61.9	62.0
4′	24.9	24.9	25.0
5′	25.7	25.7	25.7
6′	69.3	69.3	69.3
6′-Me	21.3	21.3	21.3
7-Me	22.2	22.3	22.2
4a	111.0	111.1	111.0
8a	142.5	142.5	142.5
7-CH_3CO	20.0, 169.7	20.0, 169.8	20.0, 169.8
1″	168.4	167.6	167.4
2″	122.7	112.9	127.2
3″	142.1	141.5	141.2
4″	127.0	133.8	14.7
5″	140.7	139.3	12.0
6″	19.0	14.8	
7″	12.5	11.7	



1 **2**

Fig. 1. Important ^1H–^1H COSY, HMBC and NOESY spectra of daldinins E (**1**) and F (**2**).



between (1) H-4″ and H-3″, H-5″; (2) H-5″ and H-6″. In addition, long-range correlations between H-4, H-3″, H-7″/ C-1″ were observed in its HMBC spectrum and the NOE correlation between (1) H-3″ and H-5″, (2) H-4″ and H-6″, (3) H-4″ and H-7″ in its NOESY spectrum (Fig. 1), suggesting that the substitution group at C-4 is 2-methyl-(2*E*,4*E*)-hexa-2,4-dienoyl (Ceroni and Sequin, 1982). The absolute configuration of daldinin E (**1**) was established by comparing its CD spectrum with that of daldinin C (**3**) (Hashimoto and Asakawa, 1998), which showed negative (353, 280 and 214 nm) and positive (306, 264 nm) Cotton effects. These results indicated that compound **1** possessed the same absolute configuration as daldinin C (**3**). Thus, daldinin E (**1**) was determined to be (2*E*,4*E*)-hexa-2,4-dienoic acid, 2-methyl-7*S*-(acetyloxy)-3′,4,4′,5′,6,6′,7,8-octahydro-3′-hydroxy-6′,7-dimethyl-6,8-dioxospiro[3H-2-benzopyran-3,2′-[2H]pyran]-4-yl ester.

Daldinin F (**2**) was isolated as an oil and its molecular formula was determined to be $C_{24}H_{28}O_9$ by HR-FABMS, which was the same molecular formula as **1**. Both isomeric compounds showed identical HR-MS data. The ^1H and ^{13}C NMR spectral data (Tables 1 and 2) of **2** were similar to those of daldinin C (**3**) (Hashimoto et al., 1994b; Hashimoto and Asakawa, 1998) and daldinin E (**1**) except for the signal of the substitution group at C-4. Examination of the ^1H–^1H COSY, NOESY and HMBC spectra (Fig. 1) of **2** indicated that the substitution group at C-4 is 4-methyl-(2*E*,4*E*)-hexa-2,4-dienoyl. The absolute configuration of daldinin F (**2**) was also determined by comparing its CD spectrum which showed negative (353, 276 and 221 nm) and positive (305 nm) Cotton effects with that of daldinin C. Consequently, the structure of daldinin F (**2**) was determined to be (2*E*,4*E*)-hexa-2,4-dienoic acid, 4-methyl-7*S*-(acetyloxy)-3′,4,4′,5′,6,6′,7,8-octahydro-3′-hydroxy-6′,7-dimethyl-6,8-dioxospiro[3H-2-benzopyran-3,2′-[2H]pyran]-4-yl ester.

Compounds **3** and **4** were determined to be daldinin C (Hashimoto et al., 1994b; Hashimoto and Asakawa, 1998) and 4,5,4′,5′-tetrahydroxy-1:1′-binaphthyl (BNT) (Stadler et al., 2001c), respectively. The full NMR spectral data of daldinin C (**3**) are included in Tables 1 and 2 because they had not been reported previously by Hashimoto et al. (1994b); Hashimoto and Asakawa (1998).

The occurrence of daldinins (**1–3**) in *H. fuscum* and the related *H. fuscopurpureum* (Mühlbauer et al., 2002) provides additional proof that the genera *Hypoxylon* and *Daldinia* are closely linked chemotaxonomically, albeit daldinins E and F were so far not found in the latter genus. In contrast, mycelial cultures of these fungi consistently diverge in the production of dihydroisocoumarins by the former and naphthalene and chromone derivatives by the latter genus (Stadler et al., 2001a,c).

Species of *Hypoxylon* sect. *Annulata* contain **4** as a major component, in addition to yet unknown metabolites. Within *Hypoxylon* sect. *Hypoxylon*, the presence of four chemotypes now becomes evident (Fig. 2). While *H. fuscum* shows some affinities to *D. childiae* J.D. Rogers & Y.-M. Ju, as revealed from the presence of daldinal A and daldinins besides **4**, *H. macrocarpum* Pouz. contains **4** and macrocarpones (Mühlbauer et al., 2002). Species of the third chemotype, including, e.g., *H. fragiforme* (Pers.: Fr.) J. Kickx fil., are lacking **4** and contain mitorubrins instead (Stadler et al., 2001a,c). These data are preliminary, considering that the genus is rather large and 155 taxa of *Hypoxylon* were recognized by Ju and Rogers (1996). Still, the current study emphasized the utility of secondary metabolites in segregation of *Hypoxylon* spp. and the need for further elucidation of taxonomically significant chemical components, such as the unknown components of *H. subgilvum* and sect *Annulata* detected by Mühlbauer et al. (2002).

The antioxidant activity of four compounds (**1–4**) from *H. fuscum* was tested by using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method (Blois, 1958). Their antioxidant activities were defined as the amount of antioxidant necessary to decrease the initial DPPH

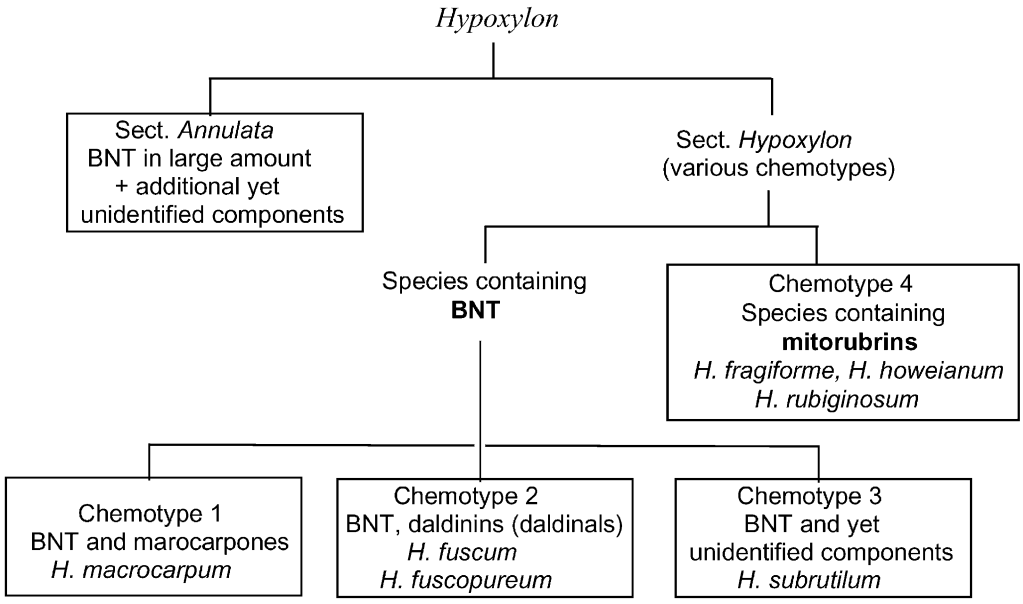


Fig. 2. Distribution of stromatal secondary metabolites in *Hypoxylon*. Compiled from Mühlbauer et al. (2002) and the results of the current study.

radical concentration by 50% [IC_{50} (μM)] and compared with those of known antioxidant ascorbic acid. The antioxidative activity of **4**, which has never before been reported in the literature, was almost the same as that of ascorbic acid with the IC_{50} values of 18.2 and 16.5 μM, respectively. In contrast, daldinins E (**1**) and F (**2**), and daldinin C (**3**) were devoid of significant activities with IC_{50} values of 178.9, 212.3 and 412.0 μM, respectively.

3. Experimental

3.1. General

Optical rotations were measured on a JASCO DIP-1000 polarimeter with $CHCl_3$ as solvent. CD spectra were measured on a JASCO J-725 spectrometer in EtOH. UV spectra were obtained on a Shimadzu UV-1650PC instrument in EtOH. IR spectra were measured on JASCO FT/IR-5300 spectrophotometer. The ^1H and ^{13}C NMR spectra were recorded on a Varian Unity 600 NMR spectrometer (600 MHz for ^1H and 150 MHz for ^{13}C), using either $CDCl_3$ or CD_3OD as solvent. Chemical shifts are given relative to TMS (δ0.00) as internal standard (^1H) and δ77.0 ppm from $CDCl_3$ and δ49.0 ppm) from CD_3OD as standards (^{13}C). Mass spectra were recorded on a JEOL JMS AX-500 spectrometer. Preparative medium-pressure liquid chromatography (MPLC) was performed with Work-21 pump (Lab-Quatec Co., Ltd, Japan) and a Lobar column (Merck). CC was carried out on Sephadex LH-20 (Amersham Pharmacia Biotech, $CHCl_3$–MeOH, 1:1). The preparative HPLC and HPGPC experiments were performed at room temperature using a Gilson Abimed (Ratingen, Germany) HPLC system.

3.2. Material

Fruit bodies of *Hypoxylon fuscum* were collected and identified by M.S. and H. Wollweber from trunks of *Corylus avellana* in the Neandertal near Haan-Gruiten, North Rhine Westphalia, Germany in February 2000 and again, from the same tree, in April 2003. Voucher specimens and a corresponding culture (deposited with CBS, Utrecht, Netherlands, as CBS 113049), which showed the characteristics of the genus and species sensu Ju and Rogers (1996) and Petrini and Müller (1986), respectively, are deposited at the Botanische Staatsammlung, Munich, Germany, and at the mycological herbarium of the Fuhlrott Museum, Wuppertal, Germany. The material was designated by Mühlbauer et al. (2002) as Ww 3723.

3.3. Extraction and isolation

Freshly collected stromata of *H. fuscum* (23 g) were detached carefully from the substrate, pulverised in a mortar and extracted with MeOH (2×250 ml) at 40 °C in an ultrasonic bath for 30 min each. The combined extracts were filtered and concentrated in vacuo to yield an oily crude product (650 mg), which was filtered through a BondElut C18 (1 g) solid phase extraction cartridge (Baker, Deventer, Netherlands) and subjected to preparative HPLC in which the stationary phase is an MZ Analysentechnik (Mainz, Germany) Kromasil C18

(250×40 mm; 7 μm), and the mobile phase consisted of 0.01% aq. TFA and MeCN. Elution at a flow rate of 10 ml/min with 50% MeCN for 30 min, followed in a linear gradient from 50 to 100% MeCN in 30 min, and then 100% MeCN for 30 min, gave three fractions (Fr. 1–3). Fraction 1 (108.7 mg) was purified by Sephadex LH-20 CC to give daldinin C (**3**) (14.1 mg) and BNT (**4**) (51.4 mg). Fraction 2 (123.9 mg) was separated by MPLC using $CHCl_3/CH_3OH/H_2O$:25/2.5/0.1 as solvent system to afford an intermediate product (59.8 mg). Aliquots of the resulting intermediate product (10 mg) were further purified using HPGPC on Merck (Darmstadt, Germany) LiChroGel PS1 (250×25 mm; 10 μm) as stationary phase and 2-propanol as mobile phase under isocratic conditions at a flow rate of 5 ml/min, yielding daldinin E (**1**) (1.27 mg) that eluted at 33–37 min and daldinin F (**2**) (2.18 mg) at 41–51 min.

3.3.1. Daldinin E (*1*)

Oil, $[\alpha]_D^{20}$ +87.7° (*c* 0.3, $CHCl_3$). HR-FABMS: *m/z* 461.1845 $[M+H]^+$, $C_{24}H_{29}O_9$ requires 461.1812. UV λ_{max} (EtOH) nm (log ε): 219 (4.0), 268 (4.3). CD (EtOH) λ_{ext} nm ($\Delta\varepsilon$) 353 (−1.17), 306 (+10.4), 280 (−0.16), 264 (+4.82), 214 (−11.55). IR (KBr) cm^{-1}: 3465, 2934, 1714, 1675, 1628, 1578, 1446, 1223, 1090, 986. ^1H and ^{13}C NMR ($CDCl_3$) (Tables 1 and 2).

3.3.2. Daldinin F (*2*)

Oil, $[\alpha]_D^{20}$ +28.9° (*c* 0.4, $CHCl_3$). HR-FABMS: *m/z* 461.1845 $[M+H]^+$, $C_{24}H_{29}O_9$ requires 461.1812. UV λ_{max} (EtOH) nm (log ε): 200 (3.9), 270 (4.1). CD (EtOH) λ_{ext} nm ($\Delta\varepsilon$) 353 (−0.80), 305 (+6.71), 276 (−0.75), 221 (−7.90). IR (KBr) cm^{-1}: 3468, 2934, 1714, 1676, 1626, 1578, 1446, 1247, 1090, 988. ^1H and ^{13}C NMR ($CDCl_3$) (Tables 1 and 2).

3.3.3. DPPH free-radical scavenging activity

The free radical-scavenging activity of daldinins E (**1**), F (**2**), C (**3**) and binaphthyl (**4**) was measured using the DPPH method (Blois, 1958). Each compound and ascorbic acid was dissolved in EtOH at different concentrations (100, 50, 10, 5 and 1 μg/ml) and mixed with DPPH (180 μl). After incubation at room temp for 20 min, absorbance at 517 nm was measured with a Spectra Max 340 PC and their IC_{50} were calculated.

Acknowledgements

We thank Dr. Hartmund Wollweber (Wuppertal, Germany) for pointing out the location of the fungus and for providing reference materials from his herbarium, as well as Dr. S. Seip (Bayer Health Care, Wuppertal) and Ms. Y. Okamoto (TBU) for recording mass spectra.

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