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Cohaerins A and B, azaphilones from the fungus *Hypoxylon cohaerens*, and comparison of HPLC-based metabolite profiles in *Hypoxylon* sect. *Annulata*

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Dedicated to Prof. Dr. Kurt Hostettmann on the occasion of his 60th birthday

Abstract

Azaphilones, named cohaerins A and B were isolated from stromata of the xylariaceous ascomycete *Hypoxylon cohaerens*. Their absolute structures were determined by spectroscopic methods (2D NMR, MS, IR, UV CD), and subsequently confirmed by acetylation. Stromatal metabolite profiles of several taxa of *Hypoxylon* sect. *Annulata* were also generated using analytical HPLC with diode array and MS detection. The cohaerins were neither found in other *Hypoxylon* spp., nor in other Xylariaceae. However, they were present even in holotype material of *H. cohaerens*, collected over 200 years ago. The binaphthalene BNT was also omnipresent in sect *Annulata*, and its derivatives, the benzo[*j*]fluoranthenes daldinone A and truncatone, as well as presumably related compounds. These fungi were found devoid of other types of azaphilone pigments of the Xylariaceae, such as mitorubrins and daldinins, the latter of which are widespread in certain groups of *Hypoxylon* sect. *Hypoxylon*. Hence, chemotaxonomic data largely support the current generic concept. The original source of truncatone was identified as *Hypoxylon annulatum*.

Keywords: Chemosystematics; Fungi; Structure elucidation; HPLC profiling

1. Introduction

Azaphilones, polyketides produced by ascomyceteous fungi (Gill and Steglich, 1987), are frequently encountered in particular genera of the Xylariaceae such as *Daldinia* Ces. & De Not., *Entonaema* A. Möller and *Hypoxylon* Bull. (Hellwig et al., 2005; Stadler et al., 2004a,b). Of these, the first azaphilones reported from this family were the mitorubrins (Steglich et al., 1974). Since then, various bioactivities were described for mitorubrins and related compounds, and they were even patented as antiprotozoan dihydrofolate reductase inhibitors (Hayashi et al., 1996). Nonetheless, the trivial name "azaphilones" relates to the ability of these

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compounds to react spontaneously with ammonium ions (Gill and Steglich, 1987) and amines. They are thus likely to exhibit broad, non-specific, activities in biological systems. In Xylariaceae fruiting bodies, they are located in granules or contained in waxy layers directly below the surface and may only be exposed to the environment if the maturing stromata are damaged. This feature may relate to their natural role as a means of chemical defence against insects and other competing organisms (Hellwig et al., 2005 and references therein).

The colours of both the aforementioned granules and their stromatal pigments in 10% KOH are determined by comparison with a botanical chart (Rayner, 1970) as well as being important diagnostic characters for species differentiation (Ju and Rogers, 1996; Ju et al., 2004). Indeed, these colours are strongly correlated with the specific occurrence of various types of metabolites (Stadler et al., 2001a, 2004a,b), suggesting a chemotaxonomic relationship. Previously, we already characterised several azaphilones, such as entonaemins, daldinins, rubiginosins and hypomiltin from *Daldinia*, *Entonaema*, *Hypoxylon*, and *Pulveria* Malloch and Rogerson (Buchanan et al., 1995; Hashimoto and Asakawa, 1998; Hellwig et al., 2005; Quang et al., 2004a,b). Concurrent HPLC-profiling studies on *Hypoxylon* and allied Xylariaceae revealed that their secondary metabolites patterns are highly species-specific, and that they are in excellent accordance with the modern taxonomic classification (Hellwig et al., 2005; Mühlbauer et al., 2002; Stadler et al., 2001a, 2004a,b). These studies also revealed a high diversity of presumably unprecedented chemical matters, suggesting that these fungi constitute yet untapped sources of metabolic diversity that are worthwhile to be further exploited.

Notably, the identification of *Hypoxylon* spp. treated previously in the literature (including reports on their chemistry) was frequently based on the monograph by Miller (1961), which is now outdated. Several taxa were recently removed from *Hypoxylon*, some species were newly circumscribed, and many new taxa designated (Ju and Rogers, 1996; Ju et al., 2004). The genus is now divided into sections *Hypoxylon* (ca. 110 species and varieties) and *Annulata* (ca. 33 species and varieties) sensu Ju et al. (2004). Most *Hypoxylon* spp. hitherto studied intensively for secondary metabolites belong to sect. *Hypoxylon*, while there is still a lack of information on the chemistry of the sect. *Annulata*. Only from one species, identified as "*H. truncatum*" (sensu Miller, 1961) collected in Japan, has truncatone and other aromatic metabolites been obtained (Buchanan et al., 1995; Hashimoto and Asakawa, 1998; Koyama et al., 2002). Furthermore, isobutyric acid derivatives that are also known to be spider pheromones were obtained from the same species (Quang et al., 2003). The taxonomy of this fungus was therefore revised in the present study (see Section 2).

Mühlbauer et al. (2002) evaluated several European species of *Hypoxylon* by application of analytical HPLC methodology, including *H. cohaerens* and *H. multiforme* of sect. *Annulata*, and detected apparently specific compounds in *H. cohaerens*. Recently, large quantities of the stromata of this species were made available, permitting its intensified mycochemical evaluation. Consequently, we here report the isolation and the absolute structures of two new azaphilones, for which we propose the names cohaerins A and B (**1**,**2**). In addition, preliminary evidence on the usefulness of HPLC-based metabolite profiles to assess the chemosystematics of related species accommodated in *Hypoxylon* sect. *Annulata* is provided.

2. Results and discussion

2.1. Isolation and characterisation of cohaerins

The MeOH extract of *H. cohaerens* was separated by reversed-phase preparative HPLC, and the resulting fractions subjected to silica gel and Sephadex LH-20 chromatography to obtain three compounds (**1–3**) as described in Section 3. Their spectroscopic analyses revealed **1** and **2** to be new fungal metabolites. A third major component (BNT, **3**) had already been detected in *H. cohaerens* by HPLC (Mühlbauer et al., 2002), and whose occurrence was confirmed in the current study by spectroscopic analysis of the purified metabolite.

Cohaerin A (**1**) gave a molecular ion peak at 459 $[M + Na]^+$ upon FAB-MS, and the high resolution FAB-MS indicated the molecular formula of $C_{26}H_{28}O_6$. This was confirmed by ESI-HPLC-MS data, revealing molecular peaks in both the positive and negative ESI modes. Its ^{1}H NMR spectrum (Table 1) revealed the presence of seven olefinic protons, two vinyl methyls, one primary methyl, and one quaternary methyl. The ^{13}C NMR spectrum showed two α,β-unsaturated ketones (δ_C 193.8, 193.6; corresponding with (γ_{max} 1704 cm^{-1} by IR), one unsaturated ester (δ_C 167.4), and one phenolic carbon (δ_C 154.5). Comparison of its spectral data with that of daldinins A–C (Hashimoto and Asakawa, 1998) and E–F (Quang et al., 2004a) suggested that **1** possessed an azaphilone structure with two different units at C-7 and C-3. Interpretation of its ^{1}H–^{1}H COSY, NOESY and HMBC spectra (Fig. 2) indicated that one unit is 2-methyl-2*E*-octenoic acid (Hasegawa, 1985); the other is 3-methylphenol. The absolute configuration of **1** at C-7 was established to be *S* by comparing its CD spectrum with that of (7*S*)-7-acetoxy-3,5,7-trimethyl-2-benzopyrane-6,8-dione (Steyn and Vleggaar, 1976), which showed positive (359 nm) and negative (275 nm, 237 nm) Cotton effects. Acetylation of **1** by acetic anhydride in pyridine resulted in the formation of the monoacetate **1a**, [Positive FAB-

Table 1
^{1}H and ^{13}C NMR spectroscopic data for compounds **1** and **2** ($CDCl_3$)

Position	Compound 1		Compound 2	
	δ_H	δ_C	δ_H	δ_C
1	8.03 (*d*, 1.4)	154.8	7.90 (*d*, 0.8)	154.3
3		155.4		153.1
4	6.45 (*s*)	114.2	6.22 (*s*)	114.4
5	5.65 (*d*, 1.1)	107.5	5.59 (*d*, 1.4)	108.1
6		193.8		193.5
7		84.1		84.2
8		193.6		193.3
9	1.63 (*s*)	22.1	1.61 (*s*)	22.0
10		118.6		130.1
11		138.9		161.2
12	6.81 (*d*, 7.7)	122.5	2.82 (*dd*, 4.1, 18.7) 2.63 (*dd*, 7.1, 18.7)	40.9
13	7.19 (*t*,7.7)	131.6	4.39 (*m*)	65.3
14	6.76 (*d*, 7.7)	113.8	2.80 (*dd*, 3.9, 16.2) 2.63 (*dd*, 7.7, 16.2)	46.0
15		154.5		193.7
16	2.29 (*s*)	20.0	2.09 (*s*)	22.9
4a		143.1		142.0
8a		115.1		115.1
1′		167.5		167.4
2′		126.0		126.0
3′	6.98 (*dt*, 1.4, 7.4)	145.8	6.89 (*dt*, 1.4,7.4)	145.6
4′	2.18 (*m*)	28.9	2.19 (*dd*, 7.4, 14.3)	28.9
5′	1.45 (*m*)	28.0	1.46 (*m*)	28.0
6′	1.31 (*m*)	31.5	1.31 (*m*)	31.6
7′	1.31 (*m*)	22.5	1.31 (*m*)	22.5
8′	0.90 (*t*, 7.1)	14.0	0.90 (*t*, 7.1)	14.0
9′	1.84 (*d*, 1.4)	12.2	1.85 (*d*, 1.4)	12.2

MS: 479 $[M + H]^+$; HR-FABMS *m/z* 479.2088 ($C_{28}H_{31}O_7$, requires *m/z* 479.2070)], indicating the presence of one phenolic hydroxyl group in the parent compound (**1**). Thus, cohaerin A (**1**) was determined to be 3-(2-hydroxyl-6-methylphenyl)-(7*S*)-7,8-dihydro-7-methyl-6,8-dioxo-6H-2-benzopyran-7-yl 2-methyl-(2*E*)-octenoate as depicted in Fig. 1.

For cohaerin B (**2**), which was concurrently obtained from *H. cohaerens*, the only notable difference to the structure of **1** is the side chain at C-3 of the azaphilone system. Interpretation of its ^{1}H–^{1}H COSY spectrum showed that H-13 was coupled to both H-12 and H-14. In addition, a methylene group (H-14) was coupled to an unsaturated ketone C-15 δ_C 193.7), and the vinyl methyl (H-16) was coupled to C-10, C-11 and C-12 in the HMBC spectrum indicating that the methyl was located at C-11. Furthermore, the H-13 attached to the oxygenated carbon C-13 (δ_C 65.3), showed HMBC correlations with H-12 and H-14. On the basic of the above spectroscopic data, the substitution part at C-3, which was confirmed by HMBC correlation between H-4 and C-10, was deduced to be 3-methyl-5- hydroxy-2-cyclohexen-1-one (Ruden and Litterer, 1975). Therefore, cohaerin B (**2**) was determined as 3-(2-methyl-4-hydroxy-6-oxo-1-cyclohexenyl)-(7*S*)-7,8-dihydro-7-methyl-6,8-dioxo-6H-2-benzopyran-7-yl 2-methyl-(2*E*)-octenoate, except for the absolute configuration at C-13, as shown in Fig. 1.

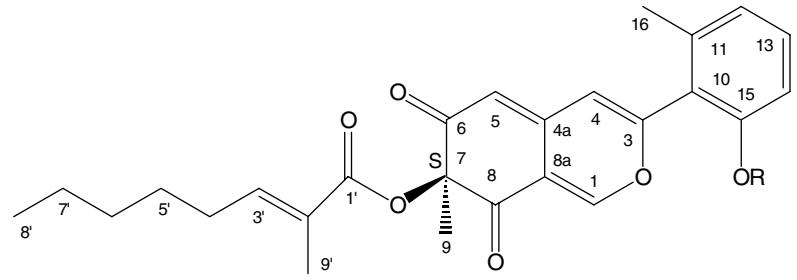
2.2. A preliminary chemotaxonomic survey of *Hypoxylon* sect. *Annulata*

In continuation of our ongoing chemotaxonomic survey of the Xylariaceae, metabolite profiles of *Hypoxylon* sect. *Annulata* were compared by HPLC-based methodology for the first time, including a significant number of materials and species. Previously identified compounds and the pure cohaerins (**1**, **2**) were employed as external and internal standards. Daldinone A (**4**) and truncatone (**5**) were also purified from representatives of sect. *Annulata*, and whose identities were confirmed by comparison of spectroscopic data. The results of the metabolite profiling study are compiled in Table 2, and the implications of these findings are discussed below.

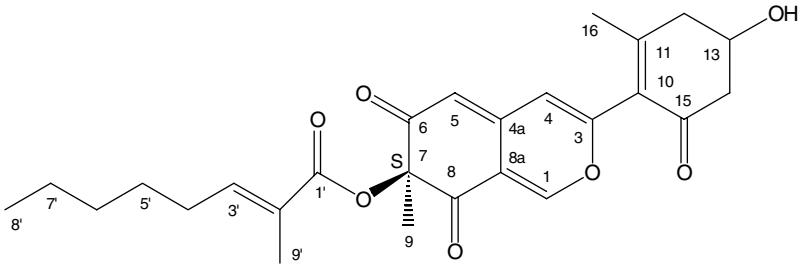
2.2.1. General characteristics of metabolite profiles in sect. *Annulata*

BNT (**3**) was the only compound detected in all examined extracts. This metabolite is widely distributed in the Xylariaceae, but appears to be rare in other fungi and has not been reported in higher plants (Stadler et al., 2001a,b, 2004a). Even in the Xylariaceae, it is not omnipresent in all of the genera where it occurs. For instance, it is not found in several species of *Hypoxylon* sect. *Hypoxylon*, and morphologically similar species in this section can even be discriminated well by HPLC, based on its occurrence (Hellwig et al., 2005; Stadler et al., 2004b). In many specimens examined, several presumably related metabolites co-occurred with BNT, as revealed from characteristic HPLC–UV/Vis and HPLC-MS data, which are suggestive of naphthalene derivatives.

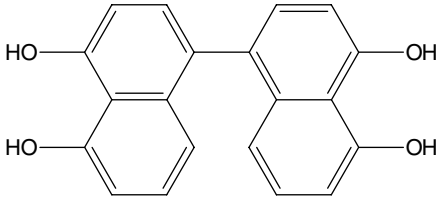
Aside from the aforementioned binaphthalenes, the macrocarpones (Mühlbauer et al., 2002; Hellwig et al., 2005) and the benzophenones of the daldinal type (Hashimoto and Asakawa, 1998; Stadler et al., 2001a,b), most other stromatal pigments that are known to occur in *Hypoxylon* constitute the azaphilones. They can be divided into three main subgroups; (i) the mitorubrin-like metabolites including mitorubrins, rubiginosins and hypomiltin, where an orsellinic acid moiety is attached to the bicyclic azaphilone backbone by an ester bond; (ii) the daldinins, constituting *spiro*-tricyclic derivatives of this core, and (iii) the cohaerins, which have a third ring system attached by a C–C bond to the bicyclic azaphilone system. Of these, the cohaerins (**1**, **2**) and the daldinins (Quang et al., 2004a) appear most closely related to one another, since they all possess a *S*-configuration at C-7. While daldinins, mitorubrins and rubiginosins can co-occur in a single species of sect. *Hypoxylon* (*H. rubiginosum*; Quang et al., 2004b), the cohaerins were not detected in any of the materials studied by Hellwig et al. (2005) and Stadler et al.



1. R= H
1a. R = Ac



2



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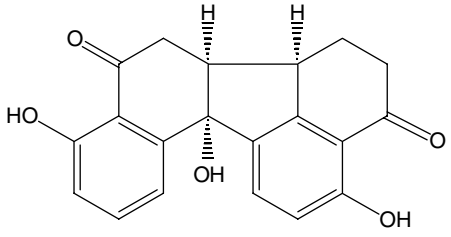

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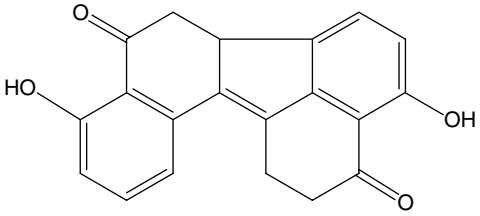

5

Fig. 1. Structures of compounds **1**–**5**.

(2004a,b) upon re-analysis of the HPLC data recorded during the previous studies of the Xylariaceae. Compounds **1** and **2** are thus not only the first azaphilones known to occur in species of *Hypoxylon* sect. *Annulata*, but also appear to be specific for *H. cohaerens* (Table 3). Several metabolite classes that are typical for certain groups of sect. *Hypoxylon* (i.e., mitorubrins, rubiginosins, macrocarpones, hypomiltin, daldinal) were apparently absent in the species examined of sect. *Annulata*. Hence, the chemotaxonomic data so far available strongly support the view of Ju and Rogers (1996), who suspected sect. *Annulata* to constitute a diverging lineage of *Hypoxylon* that may eventually be recognised as a separate genus.

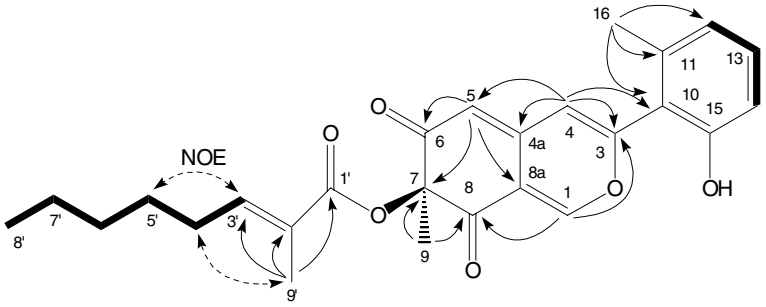

Fig. 2. Important ^{1}H–^{1}H COSY (bold lines), NOESY and HMBC (arrows) correlations of **1**.

Table 2
Distribution of secondary metabolites and KOH-extractable stromatal pigments (Ju and Rogers, 1996; Ju et al., 2004) in *Hypoxylon* sect. *Annulata*

Species/variety	Pigments in 10% KOH	Metabolites detected by HPLC				Remarks
		BNT (**3**)	Cohaerins (**1**,**2**)	Daldinone A (**4**)	Truncatone (**5**)	
Hypoxylon annulatum	GO	+/++	–	–	++	A, B, C
H. archeri	GO	+	–	–	–	C, E
H. atroroseum	GO	(+)	–	–	–	D, E
H. bovei	GO	+	–	++	–	C, H
H. bovei var. *microspora*	GO	++	–	+	++	C
H. cohaerens	GO or dilute isabelline (65)	++	++	–	–	C, D, J
H. cohaerens var. *microsporum*	Red or vinaceous	+/(+)	–	–	–	C, D
H. gombakense	Red–orange	(+)	–	–	–	C, D
H. hians	none	(+)	–	–	–	C, E
H. ilanense	Red–orange	++	–	–	–	C, D
H. leptascum var. *macrosporum*	GO	++	–	–	–	C, D
H. michelianum	Yellow–green	(+)	–	–	–	C, D
H. moriforme	GO	+	–	(+)	+/++	C, D
H. moriforme var. *microdiscus*	GO	++	–	–	–	C, G
H. multiforme	Red–orange	++	–	–	–	C, D
H. multiforme var. *alaskense*	Red–orange	++	–	–	–	C, D, F
H. nitens	GO	++	–	–	++	C
H. nothofagi	GO	++	–	–	–	C
H. pyriforme	GO	(+)	–	++	–	H
H. piirpureonitens	Vinaceous	++	–	–	–	C
H. stygium	GO	(+)/+	–	+/++	(+)/++	K
H. stygium var. *annulatum*	GO	+	–	++	(+)	H
H. thoursianum	GO	++	–	–	++	B, C
H. thoursianum var. *macrosporum*	GO	++	–	–	++	B, C, F
H. truncatum (BPI specimens)	GO	+/(+)	–	–/+	–/+	B, C
H. truncatum (JDR specimen)	GO	+	–	–	++	B, C
H. cf truncatum (La Reunion)	GO	++	–	–	–	C
H. wceolatum	Vinaceous	(+)	–	–	–	C

Legends: Intensity of peaks (HPLC–UV; 210 nm): (+): less than 2% UV absorption of total extract; only detected unambiguously by HPLC-MS; +: 5–10% total UV absorption; ++: more than 10% UV absorption of total extract; – neither detected by HPLC–UV/Vis nor by HPLC-MS. Colour codes (according to Rayner, 1970): GO: greenish–olivaceous (90), dark green (21), or dull green (70); Red–orange: orange (7), sienna (8), or apricot (42); Red: livid red (56), brick (59), or bay (6); Vinaceous: vinaceous (57), vinaceous purple (101); vinaceous gray (116). Yellow–green: citrine green (67); amber (47); honey (64). Remarks: (A) isobutyric acid type spider pheromones (Quang et al., 2003; detected by GC-MS. (B) additional major metabolites with trancatone-like HPLC characteristics (UV spectra) detected. (C) additional metabolites with BNT-like HPLC characteristics (MS, UV/Vis spectra) detected. (D) additional major metabolites with cohaerin-like characteristics (MS, UV/Vis spectra) detected; (E) only old or overmature specimens available for studies studied, yielding little extractable material. (F) HPLC profile like typical variety. (G) HPLC profile significantly deviating from typical variety. (H) Daldinone A (**4**) by far prevailing metabolite. (J) young stromata of this species yield purple pigments in KOH. (K) truncatone (**5**) only detected by HPLC-MS in specimen from Malaysia.

2.2.2. Secondary metabolite profiles of H. cohaerens and its immediate allies

Mature stromata of sect. *Annulata* are mostly melanised, while most of the species of sect. *Hypoxylon* usually have coloured stromata even at maturity (Ju and Rogers, 1996). *H. cohaerens* is one of the exceptions in sect. *Annulata* where even mature stromata retain a dark brown, rather than melanised, surface. Before material was collected for preparative work, the fungus was observed growing in the field. Various samples were taken

Table 3
Materials examined: abbreviations of herbaria follow Holmgren et al. (1990)

Hypoxylon annulatum (Schwein. : Fr.) Mont.
Japan: Shikoku, Tokushima City Park, *Quercus* stump, leg. M. Stadler and T. Hashimoto, 12 Oct. 1999 (Ww 3641, WUP)
Japan: Shikoku, Tokushima, bark *of Quercus*, July 1999, leg. Y. Asakawa & T. Hashimoto (Ww 3596, WUP)
Japan: Shikoku, Tokushima, bark *of Quercus,* July 1999, leg. Y. Asakawa & T. Hashimoto (STMA 04095, WUP)
Japan: Sendai, 12 Oct. 1912, leg. A. Yasuda, del. J.H. Miller as *H. truncatum,* rev. Ju and Rogers (1996), BPI 716885 (Lloyd herb. No 11367)
USA: Florida, Bradenton, Dec. 1994, leg. L. Cards, (JDR)
USA: Ohio, Toledo, leg. W. L. Lowater, del. J.H. Miller as *H. truncatum*, rev. Ju and Rogers (1996), BPI 716884 (Lloyd herb. No 10610), type of Hypoxylon *circumscribum* C.G. Lloyd
USA: Virginia, Arlington, bark of *Quercus alba*, 21 Mar. 1920, leg. C.L. Shear, det. J.H. Miller as *H. truncatum* (BPI 593995)
USA: Virginia, Arlington, bark of *Quercus alba*, 25 Oct. 1925, leg. C.L. Shear, det. J.H. Miller as *H. truncatum* (BPI 593994)

Hypoxylon archeri Berk
Australia: Tasmania, decorticated wood, leg. Archer, ex herb. Berkeley, K(M) 123166, holotype of *H. archeri*
New Zealand: Auckland Islands, *Metrosideros umbellata*, leg. R. Leschen, det. P. R. Johnston (PDD 72439)
New Zealand: Auckland Islands, *Metrosideros umbellata*, 21 Marc. 200, leg. & det. P. R. Johnston PRJ AK25 (PDD 72411)

H. atroroseum J.D. Rogers
Gabon: near Libreville, 11 Sep. 1980, leg. G. Gilles, sent by F. Candoussau (JDR)

Hypoxylon bovei Speg.
New Zealand*:* Buller, *Nothofagus menziesii*, 4 Oct. 1998, leg. & det. P. R. Johnston (PDD 70050)
New Zealand*:* Gisborne, *Nothofagus fusca*, 9 June 2004, leg. P. R. Johnston, det. B. C. Paulus (PDD 81112)
New Zealand*:* Mid Canterbury, *Nothofagus menziesii*, 10 May 1998, leg. & det. P. R. Johnston (PDD 70049)

Hypoxylon bovei Speg. var. *microspora* J.H. Miller
Brazil: São Paulo, on *Piptadenia communis*, leg. Puttemans 1293, type of *Hypoxylon piptadeniae* Henn., rev. Ju and Rogers (1996), S-F10644.
Philippines: Luzon Island, Laguna Province, Mt Maquiling, 28 Mar. 1914, leg. C. F. Baker, type of *Hypoxylon marginatum* var. *mammiforme* Rehm, rev. Ju and Rogers (1996), S-F10645 & S-F10693 (2 packets)
Taiwan: Nan-tou Co., Ren-ai, Tsuei-Fong, on dead wood, 23 Sep. 2002, leg. Y.-M. Ju 91092328, (HAST, JF)
Taiwan: I-lan Co., Yuan-shan, Fu-shan, on dead wood, 12 Nov. 2002, leg. Y.-M. Ju 91111212 (HAST, WUP), subjected to prep. HPLC
USA: Florida, Sanford, Aug. 1929, leg. S. Rapp, det. J.H. Miller as *H. truncatum*, rev. Ju and Rogers (1996),BPI 716887 (Lloyd herb. No. 10669)

Hypoxylon cohaerens (Pers.: Fr.) Fr.
France: Ariége, Rimont, Grand Bois, on bark of *Fagus sylvatica*. 07 Mar. 2003, leg. J. Foumier, JF-03041 (WUP)
Locality unknown: wood (L 910,270-92, lectotype, (selected by Miller, 1961) of *Sphaeria cohaerens*
Locality unknown: wood (L 910,270-61, syntype of *Sphaeria cohaerens*
Germany: Badenia-Württemberg, Bezirk Tübingen, east of Weingarten. beech grove "Jägerhölzle", *Fagus sylvatica*, 10 Mar. 2003, leg. D. Peršoh 0013 (M-0066231)
Germany: Badenia-Württemberg, Stuttgart-Feuerbach, *Fagus*, 23 Apr. 1974, leg. H. O. Baral as *H. multiforme* (STU 10290)
Germany: North Rhine Westphalia, Gruiten, *Fagus sylvatica*, 02 Apr. 2001, leg. H. Wollweber Ww 4317 (WUP), subjected to preparative HPLC
Germany: North Rhine Westphalia, Wuppertal, *Fagus sylvatica*, 02 Apr. 2001, leg. H. Wollweber Ww 3902 (WUP)
Germany: North Rhine Westphalia, Wuppertal, *Quercus rubra*, 19 Apr. 1998, leg. H. Wollweber Ww 3328 (WUP)
Germany: Saxonia, Crimmitschau, Sahnpark, *Fagus*, 23 Feb. 1997, leg. M. Graf as *H. multiforme* (GLM 38248)

Hypoxylon cohaerens (Pers.: Fr.) Fr. var. *microspora* J. D. Rogers & Candoussau
Colombia: Dpto. Cundinamarca, Boyaca, El Bosque de Las Mercedes, wood, 29 June 1974, leg. K. P. Dumont et al. CO-150 (JDR)
France: Charente Maritime: Saint Martin en Re, les Sallieres, 28 Apr. 2004, on bark of *Quercus ilex*, leg. J. Fournier JF-04065 (WUP)
France: Ariége (09): Rimont, Las Muros, on bark of *Quercus robur*, 08 Feb. 2004, leg. J. Foumier JF-04014 (WUP)
Portugal: Ilha de Madeira, Levada da Sawa de Faial (790 m), 23 Apr. 2004, *Laurus azorica*, leg. W. Jäger (STMA 04W19, WUP)
Taiwan: Nan-tou Co., Ren-ai, Tsuei-Fong, on dead wood, 23 Sep. 2002, Y.-M. Ju 91092302 (HAST, WUP).

Hypoxylon gombakense M.A. Whalley, Y.-M. Ju, J.D. Rogers & A.J.S. Whalley
Malaysia: Selangor Prov., Gombak, 23 Feb. 1993, leg. M. A. Whalley GA 13–93, det. Whalley et al. (2000), holotype (WSP)

Hypoxylon hians Berk. & Cooke
Australia: *Tasmania*, on wood, May 1852, leg. D. D. Mary (ex herb. Berkeley) K(M) 123168, part of holotype
Australia: *Nothofagus cunninghamii*, 8 Oct. 1997, leg. & det. P. R. Johnston PRJ AU97-74 (PDD 69035)

Hypoxylon leptascum Speg. var. *macrosporum* Y.-M. Ju & J.D. Rogers
Venezuela: Cerro de la Neblina, decorticated wood, 1985, leg. A. Rossman 2258–439 (JDR).

Hypoxylon michelianum Ces. & De Not.
Portugal: August 1905, leg. J. Rick, type of *Hypoxylon albotectum* Rehm, rev. Ju and Rogers (1996), S-F10696
Spain: Vigo, Isla de Cortegada, Villa Garcia, on *Laurus nobilis*, 21 Nov. 1985, leg. & det. F. Candoussau FC 5285-4 (JF-02023)

Hypoxylon moriforme Henn.
France: Caribbean, Guadeloupe Island, Les Saintes, Terre de Bas, Etangs, 15 Jan. 1994, leg. Jean Vivant, comm. F. Candoussau (JF-01190)
New Zealand: Westland, *Nothofagus* forest, 9 May 2002, leg. & det. P. R. Johnston (FDD 75691)
Samoa: Upola, leg. Reinecke, lectotype of *H. moriforme* (selected by Ju and Rogers, 1996), S-F10705
Sri Lanka: Beruwela, Nov. 2003, leg. M. Eckel (STMA 03123, WUP)

Table 3 (*continued*)

Hypoxylon moriforme Henn. var. *microdiscus* Y.-M. Ju & J.D. Rogers
Taiwan: Ping-tung Co., Hen-chuan, Ken-ting, on dead wood, 26 Nov. 2000, leg. Y.-M. Ju & H.-M. Hsieh 89112614 (HAST).
Taiwan: Taipei City, Nankang, campus of Academia Sinica, on dead wood, 17 Jan. 2002, leg. Y.-M. Ju YMJ 91011701 (HAST)
USA: Hawaii, McKenzie Park, 24 Nov. 1998, leg. J.D. Rogers (JDR)

Hypoxylon multiforme (Fr.: Fr.) Fr.
Czech Republic: Moravia, Mäahrisch Weisskirchen", Hollenschlucht, Podhorn, *Fagus sylvatica*, 28 Aug. 1912, leg. and det. F. Petrak (Flora Bohemiae et Moraviae 131), as *Hypoxylon luridum* Nitschke, K(M) 123170
France: Ariége, Alzen, Pare Ecomusee, 03 June. 2004, on bark of *Betula pendula*, leg. B. & M. Stadler (STMA- 04051, WUP)
France: Ariége, Rimont, Grand Bois, 07 Mar. 2003, on bark of *Betula pendula*, leg. J. Foumier JF-03039 (WUP)
Germany: Badenia-Württemberg, Eschach, Götzenbachtal, *Corylus*, Autumn 1974, leg. Payerl, det. H. O. Baral (STU K 665/74)
Germany: Badenia-Württemberg, Freiburg im Breisgau, *Betula*, May 1998, leg. H.-V. Tichy (Ww 3338, WUP)
Germany: Badenia-Württemberg, Freiburg in Breisgau, *Betula*, May 1998, leg. H.-V. Tichy (Ww 3341, WUP)
Germany: Badenia-Württemberg, Untergröningen, Neumühle, on cf. *Alnus,*, March 1974, leg. & det. Payerl (STU K 661/74)
Germany: Badenia-Württemberg, vicinity of Weingarten, Forest "Dickenwalwald" near Irmtobel, *Alnus* sp., 20 Mar. 2004, leg. D. Peršoh 0017 (M)
Germany. Bavaria, Munich, Allach, *Carpinus betulus*, 4 Dec. 1996, leg. & det. L. Beenken, (Triebel, Microf. Exs. 189, M)
Germany: Mecklenburg-Vorpommern, Rügen Island, Nonnevitz, mixed forest, *Sorbus aucuparia*, 14 Sep. 1980, leg. Ch. Stark, det. H. Gottschalk (GLM 07813)
Germany: Mecklenburg-Vorpommern, Zarrentin near Molln, *Salix*, 12 Jan. 1991, leg. K. Richter, det. H. Gottschalk (GLM 25194)
Germany: North Rhine Westphalia, Gruiten, angiosperm wood, 27 May 2003, leg. H. Wollweber (Ww 4330, WUP)
Germany: North Rhine Westphalia, Gruiten, *Betula pendula*, 21 Nov. 1992, leg. H. Wollweber Ww 2174 (WUP)
Germany: North Rhine Westphalia, Gruiten, *Betula*, 02 Mar. 1997, leg. H. Wollweber Ww 3103 (WUP)
Germany: North Rhine Westphalia, Gruiten, *Corylus*, 06 May 2003, leg. H. Wollweber Ww 4318 WUP)
Germany: North Rhine Westphalia, Gruiten, *Corylus*, 25 Apr. 2003, leg. H. Wollweber Ww 4315 (WUP)
Germany: North Rhine Westphalia, Wuppertal, *Alnus*, 14 Feb. 1998, leg. H. Wollweber Ww 3271 (WUP)
Germany: North Rhine Westphalia, Wuppertal, *Betula*, 21 May 1998, leg. H. Wollweber Ww 3326, Ww 3327 (WUP)
Germany: North Rhine Westphalia, Wuppertal, *Betula*, Jan. 1994, leg. H. Wollweber Ww 2366 (WUP)
Germany: North Rhine Westphalia, Wuppertal, *Betula*, Nov. 1992, leg. H. Wollweber Ww 2180 (WUP)
Germany: North Rhine Westphalia, Wuppertal, *Sorbus*, 06 June 2003, leg. H. Wollweber Ww 4347 (WUP)
Germany: Saxonia, Brandis, Kohlenberg-Teich, *Betula*, 27 Sep. 1991, leg. & det. P. Otto (GLM 28453)
Germany: Saxonia, Daubitz, NSG "Niederspreer Teichgebiet", *Populus tremula*, 19 Oct. 1990, leg. I. Dunger, det. H.-J. Hardtke (GLM 24716)
Germany: Saxonia, Markersdorf near Görlitz, *Alms*, 30.04.1982, leg. Ch. Stark, det. H. Gottschalk (GLM 11043)
Germany: Saxonia, Pulsnitz, *Salix*, 10. Feb.1989, GLM 20770, leg. E. Herschel, det. H. Gottschalk (GLM 20770)
Germany: Saxonia, Zeissholz, natural reserve "Dubringer Moor", *Rhamnus*, 28.07.1988, leg. & det. G. Zschieschang (GLM 19193)

Hypoxylon multiforme (Fr.: Fr.) Fr. var. *alaskense* Y.-M. Ju & J.D. Rogers
USA: Alaska, P. W. Island, Kasaan Bay, 1.8.1950, corticated wood of *Alnus sitchensis*, leg. J. A. Klein No. 43, det. Ju and Rogers (1996), WSP 26287, holotype of *H. multiforme* var. *alaskense*

Hypoxylon nitens (Ces.) Y.-M. Ju & J.D. Rogers
Taiwan: Taipei City, Nankang, on dead wood, 21 Mar. 2002, leg. J.-R. Guu, det. Y.-M. Ju 91032103 (HAST)
USA: St. John (US Virgin Islands), 5 Jan. 1993, leg. D. J. Lodge (JDR)

Hypoxylon nothofagi Y.-M. Ju & J.D. Rogers
New Zealand: Bay of Plenty, Mangorewa Gorge, wood of *Nothofagus menziesii*, 20 Mar. 1963, leg. J. M. Dingley as *H. rubiginosum*, rev. Ju and Rogers (1996), PDD 21889, holotype of *H. nothofagi*

H. purpureonitens Y.-M. Ju & J.D. Rogers
Brazil: Serra Araca, corticated wood, 10–13 March 1984, leg. G. J. Samuels No. 808, det. Ju and Rogers (1996), WSP 69635, isotype of *H. purpureonitens.*

H. pyriforme Y.-M. Ju & J.D. Rogers
Venezuela: Cerro de la Neblina, corticated wood, 1985, leg. A. Rossman, det. Ju and Rogers (1996), WSP 69634, isotype of *H. pyriforme*

Hypoxylon stygium (Lév) Sacc.
Gabon: Sibange, 17 Sep. 1884, leg. Büttner, type of *Hypoxylon annuliforme* Rehm, rev. Ju and Rogers (1996), S- F10764
México: Chiapas state, Ocosingo municipality, Ejido Pico de Oro, 3 June 1988, leg. F. San Martín 879 (JDR)
México: Tuxtla Gutiérrez municipality, El Sumidero Canyon, 22 May 1988, leg. F. San Martín 715 (JDR)
Taiwan: Tai-tung Co., Lan-yuh, on dead wood, 19 Oct. 2002, leg. Y.-M. Ju 91101906 (HAST); subjected to preparative HPLC

Hypoxylon stygium (Lev) Sacc. var. *annulatum* (Rehm) Y.-M. Ju & J.D. Rogers
France: Pyrénées Atlantiques, Osserain, Bel Air, 07 Nov. 2003, on bark of *Castanea sativa*, leg. J. Fournier JF 03230 (WUP)
France: Pyr. Atlantiques, Auterrive, He du Gave d'Oloron, 2 June 2004, leg. M. Stadler & J. Fournier, STMA 04058 (WUP)
Russia: "Prov. Batum, Cehis-Dzisi Caucasiae, in horto Penkov", corticated wood of *Carpinus*, 20 Feb. 1912, leg. G. Newodowski. 77, type of *Nummularia annulata*, rev. Ju and Rogers (1996), S-F10761

Hypoxylon thoursianum (Lév.) C.G. Lloyd
Tanzania: East Usambara, amani, Aug. 1903, leg. F. Eichelbaum, type of *Hypoxylon amaniense* Henn., rev. Ju and Rogers (1996), S-F19772
USA: California, Big Sur, Sudden Oak Death, wood of *Lithocarpus densiflorus*, July 2003, leg. M. Stadler STMA 03098 (WUP, JDR)

(*continued on next page*)

Table 3 (*continued*)

USA: California, Monterey, Asilomar Conference Grounds, dead but standing *Lithocarpus densiflorus*, July 2003, leg. M. Stadler, STMA 03097 (WUP)
USA: California, Redwood National Park, cf. *Quercus*, 11 Sep. 1991, leg. E. Hinrichs, herb. D. Benkert (B)
USA: California, Pasadena, Mar. 1894, A.J. McClatchie, BPI 738741, isotype of *Hypoxylon occidentals* Ellis & Everhart, rev. Ju and Rogers (1996)

Hypoxylon thoursianum (Lév.) C.G. Lloyd var. *macrosporum* San Martín, Y.-M. Ju & J.D. Rogers
México: Chiapas state, La Trinitaria municipality, Montebello Lagoons, 25.5.1988, leg. San Martin 845, det. Ju and Rogers (1996) (WSP 69642, isotype of *H. thouarsianum* var. *macrosporum*.

Hypoxylon truncatum (Schwein.: Fr.) J.H. Miller
France:La Réunion Island, Notre Dame de la Paix, west slope of the volcano, 08 Apr. 2001, leg G. Gilles, comm. F. Candoussau, det. J. Fournier (JF-01072) as *H.* cf. *truncatum*. Tropical form with deviating ascospores as described in Ju and Rogers (1996)
Singapore: 27 DEC. 1919, leg. T. F. Chipp, det. J.H. Miller, conf. Y.-M. Ju, BPI 716880 (Lloyd herb. 11325)
USA: Missouri, Perryville *Quercus alba*, no date, leg. C. H. Demetrio (Rabenhorst-Winter Fungi Europaei 3668, as *Sphaeria annulata)*, det. J.H. Miller as *H. truncatum* (BPI 593996)
USA: North Carolina, Durham Co., Hill Forest, Grand Vernia, 18 May 2000, leg. J. D. Rogers (JDR)
USA: North Carolina, Salem, *Quercus alba*, 26 Feb. 1913, leg. C.L. Shear, det. J.H. Miller as *H. truncatum* (BPI 593991)
USA: North Carolina, Winston-Salem, *Quercus alba*, 19 May 1934, leg. C.L. Shear, det. J.H. Miller as *H. truncatum* (BPI 593999)
USA: Virginia, Dunn Loring, *Quercus alba*, 25 Oct. 1918, leg. C.L. Shear, det. J.H. Miller as *H. truncatum* (BPI 593992)

USA: Virginia, Dunn Loring, *Quercus alba*, 3 Mar. 1918, leg. C.L. Shear, det. J.H. Miller as *H. truncatum* (BPI 593993)
USA: Virginia, Oakton, *Quercus alba*, 15 Nov. 1918, leg. C.L. Shear, det. J.H. Miller as *H. truncatum* (BPI 593998)
Locality unknown:det. J.H. Miller as *Hypoxylon truncatum*, conf. Y.-M. Ju, BPI 716881 (Lloyd herb. 11422)
Locality unknown:leg. Langlois, 22 Feb. 1892, det. J.H. Miller as *Hypoxylon truncatum*, conf. Y.-M. Ju, BPI 716883 (Lloyd herb. 11435)

Hypoxylon urceolatum (Rehm) Y.-M. Ju & J.D. Rogers
Philippines:Luzon Island, Laguna Prov., Mt. Maquiling, 22 Sep. 1912, leg. C. F. Baker 70, type *of Nummularia urceolata*, rev. Ju and Rogers (1996), holotype of, *urceolatum*, S-F10792
Taiwan:Nan-tou Co., Yu-chee, Lien-hwa-chee, on dead wood, 26 Nov. 2001, leg. Y.-M. Ju 90112618 (HAST,WUP)

JDR refers to the personal herbarium of Dr. Jack D. Rogers, Pullman, WA (USA) and YMJ to the personal herbarium of Dr. Yu-Ming Ju, Taipei, Taiwan. They also identified these specimens, including all materials designated HAST and WSP. Other materials listed were identified by the authors if not indicated otherwise. Herbarium codes follow Holmgren et al. (1990).

throughout the vegetation period, checked microscopically and analysed concurrently by HPLC. Cohaerins were formed concurrently or slightly before ascospore formation, while young stromata bearing the conidial stage yielded no cohaerins but contained instead different, yet unidentified, compounds. When harvested for extraction, most of the stromata had just begun to form asci and ascospores. Overmature stromata collected in winter only yielded 5–10% of cohaerins as judged from HPLC analyses in comparison with mature material, but the overall composition of the metabolite profile had only changed slightly. Obviously, the cohaerins are contained preferably in the dark brown granules located below the stromatal surface in *H. cohaerens*. Extracts from these granules prepared under a dissecting microscope yielded extraordinarily high amounts of cohaerins (**1**, **2**) and less BNT (**3**) than extracts prepared concurrently under normal conditions from the same specimens.

We were kindly permitted to withdraw minute amounts of pigment granules from the type material of *Sphaeria cohaerens* by the curators of the Nationaal Herbarium Leiden (L). Even though the exact collection date is not known for this specimen, it had been definitely collected prior to 1797, when first described by the famous mycologist, Christiaan Hendrik Persoon. As established by HPLC-MS, the extract prepared from these granules readily yielded cohaerins A and B, as well as BNT (**1**–**3**). These observations, as well as previous work by Stadler et al. (2004a) and further data presented in the current study, confirmed that the metabolites of *Hypoxylon* spp. and other Xylariaceae can remain stable in herbarium specimens for centuries, provided that the materials are well preserved.

Several specimens of two European taxa of sect *Annulata* were studied, both of which appear to be closely related to *H. cohaerens* as judged from morphological characters (Ju and Rogers, 1996): *H. multiforme* and *H. cohaerens* var. *microspora*. The two varieties of *H. cohaerens* are not regarded as separate species, since they mainly differ in their ascospore size and in the colours of their stromatal pigments (Rogers and Candoussau, 1980; Ju and Rogers, 1996) and otherwise show very similar morphological characters of both their sexual and asexual stages. The typical variety of *H. cohaerens* is highly associated with beech wood and almost exclusively occurs on the Fagaceae in the Northern temperate zone, while its small-spored counterpart is found in warmer climates, including tropical as well as temperate regions. Indeed, all specimens of *H. cohaerens* var. *microspora* showed quasi-identical secondary metabolite profiles, no matter whether they were found in America,

France, Madeira or Taiwan. However, they did not contain cohaerins, and some of their unknown main constituents were also found in *H. multiforme*, *H. michelianum*, *H. leptascum* var. *macrosporum* and *H. urceolatum*. The last two of the latter species appear unrelated to the remainder of these taxa but show high morphological similarities to one another: The typical variety of *H. leptascum*, which was not yet studied by HPLC profiling, mainly differs from *H. urceolatum* in its KOH-extractable pigments (greenish-olivaceous in the former and purple in the latter), while *H. leptascum* var. *macrosporum* has in addition larger ascospores than *H. urceolatum*. (Ju and Rogers, 1996). Accordingly, *H. urceolatum* contained larger amounts of binaphthalene-like metabolites while *H. leptascum* var. *macrosporum* contained more of the compounds that we suspect to be azaphilones as judged from their HPLC characteristics.

H. multiforme, a species highly associated with Betulaceae, is perhaps the most common member of sect. *Annulata* in Europe, and appears morphologically closely related to *H. cohaerens*. Several materials of this species were available for comparison. In all of them, cohaerins (**1**, **2**) were also absent, but metabolites with similar HPLC characteristics (see below) were detected especially in young and recently collected stromata. In old and overmature materials, these unknown compounds were also observed; however, the amounts detected in comparison to BNT substantially declined.

H. multiforme showed nearly identical metabolite profiles as its variety, *H.multiforme* var. *alaskense*. Both yielded higher concentrations of BNT as the aforementioned species, while those contained instead apparently specific unidentified metabolites with HPLC characteristics similar to that of BNT (data not shown). Aside from *H. leptascum* var. *macrosporum*, these species all deviate from the majority of *Hypoxylon* sect. *Annulata* in not having greenish–olivaceous stromatal pigments (Table 2). All were also devoid of compounds **4** and **5** (see below). In all aforementioned species and in both varieties of *H. moriforme*, metabolites with similar HPLC–UV/Vis spectra and slightly larger masses as deduced from HPLC-MS (estimated mw between 460 and 500 Da) to the cohaerins were detected. These compounds may thus constitute further azaphilone derivatives, rather than binaphthalenes. Currently, preparative work is ongoing to establish their identity.

2.2.3. Species containing benzo[j]fluoranthenes (*4* and *5*)

The remaining species studied were apparently devoid of cohaerins and presumably related compounds. At this time only a little information is available as to the correspondence of various yet unknown metabolites in their extracts. However, at least two of these constituents were available as standards, and both were proven to be contained in various taxa and to constitute oxidised derivatives of BNT (**3**). In any case, the biogenesis of truncatone (and thus daldinone) from BNT (**3**) would be easy to conceive (Hashimoto and Asakawa, 1998; Gill, 2003), and it is therefore not surprising to find the co-occurrence of such compounds in further species of BNT-containing Xylariaceae.

For instance, both varieties of *H. stygium* contained a main metabolite, whose HPLC characteristics had been reported before (as ''HI 1'') by Hellwig et al. (2005) from taxa of *Hypoxylon* sect. *Hypoxylon*. In the current study, it was also found in both varieties of *H. bovei*, *H. moriforme*, and other species listed in Table 2. By comparison with an authentic sample, daldinone A (**4**), previously isolated from *Daldinia* (Quang et al., 2002), proved to be identical with this compound. This was also confirmed by its isolation and characterisation from *H. stygium*. Compound (**4**) is thus present in some species of both sections of *Hypoxylon*, as well as in *Daldinia*. Further work will show whether its occurrence can serve as a parameter in the segregation of morphologically related species.

Truncatone (**5**) was first reported by Buchanan et al. (1995) and Hashimoto and Asakawa (1998) from a Japanese *Hypoxylon* sp. identified as *H. truncatum* ss. Miller (1961). The original specimen used for isolation and two further materials collected in the same geographic region were now identified as *H. annulatum* sensu Ju and Rogers (1996). *H. annulatum* and *H. truncatum* are somewhat similar, but aside from morphological differences, it had even proven by PCR-based methodology (Yoon and Glawe, 1993), that they are different species. This fungus, which was later also studied by Quang et al. (2003) is here recognised as *H. annulatum*, due to the relatively large semiglobose stromata, lacking prominent perithecial mounds and with other characteristics as defined in the above monograph. Interestingly, *H. truncatum* sensu Ju and Rogers (1996) notably still remains to be identified from Asia. All three Japanese specimens of *H. annulatum* contained not only truncatone (**5**) and its derivatives, but the isobutyric acid type spider pheromones were also detected in their extracts by GC-MS using the experimental procedure described by Quang et al. (2003). No other *Hypoxylon* sp., including *H. truncatum*, contained these pheromones, according to preliminary results on representative specimens.

Truncatone (**5**) was identified by HPLC in several specimens listed here as *H. annulatum*, including some type and other authentic specimens. The compound was also found in various other species of sect. *Annulata* (Table 2), and isolated from *H. bovei* var. *microspora* by preparative HPLC. However, it proved absent in various specimens identified as *H. truncatum* ss. Ju and Rogers (1996), except for the recently collected material from USA in the JDR herbarium. While this specimen showed a similar HPLC profile as the recently collected *H. annulatum*, truncatone was not unambiguously

detected even in some old specimens of the latter species. The results were less consistent as in other species groups, and their genuine metabolites may have decayed to some extent. Truncatone (**5**) was found unstable upon heating to 80 °C for 15 min and may therefore not have survived such procedures that have been widely employed for preservation of herbarium materials in the past. Additional studies, preferably using recently collected materials, should be carried out to show whether *H. annulatum* and *H. truncatum* can be separated by characters relating to their pigment chemistry.

Truncatone (**5**) appears to be chemically and biogenetically related to daldinone A (**4**) (Gill, 2003) and indeed co-occurs in some of the species studied here (Table 2). Therefore, it is likely that both compounds and even further derivatives may be eventually encountered upon preparative work even in those species where only one of them was detected in the current study. Both compounds gave a greenish–olivaceous colour when incubated in 10% KOH and are most probably involved in the taxonomically significant colour reactions of *Hypoxylon*. Such pigment colours are characteristic of various species in the sect. *Annulata* (Table 2 and Ju and Rogers, 1996), including *H. cohaerens*, which was devoid of compounds **4** and **5**. Hence, greenish–olivaceous pigments in this section of the genus may at least be caused by two different types of metabolites: i.e. azaphilones and naphthalene derivatives. The material from Reunion island (JF-01072) belongs to the specimens of *H. truncatum* that differ from typical material in their host-specificity (not associated with *Quercus*), geographic distribution (tropical) and in ascospore size (Ju and Rogers, 1996). A comparison of the metabolite profiles of JF-01072 with recently collected material of *H. truncatum* (JDR specimen from USA) showed significant differences. BNT(**3**) and its putative derivatives were the main detectable metabolites, but no truncatone (**5**) was contained in JF-01072, implying that this tropical *H. truncatum*-like fungus may indeed deserve to be eventually recognised as a separate taxon. As shown in Table 2, there are other examples that varieties erected on the basis of ascospore size appear to differ significantly from one another in their HPLC profiles. Further studies, including morphological data and PCR-based methodology, may eventually clarify whether the differences in chemotypes will lead to discovery of further species. However, before this can accomplished, the results of the current study need to be refined. This is because some of the fungi studied were old, and other materials were apparently overmature; hence, such materials yielded only minute amounts of extractable pigments, although this is actually a general feature of this section. Even though the characteristic metabolites of *H. cohaerens* have endured preservation for over 200 years in the holotype, this may not be the case with other materials, e.g., if those have been subjected to excessive heat treatment (see above for truncatone and *H. truncatum*). The lack of such compounds is therefore certainly less significant in chemotaxonomic studies than their presence (see also Stadler et al., 2004a), and the formation of artefacts should always be excluded by concurrent studies on freshly collected specimens. HPLC profiling can only give hints to mycologists where to look for new taxa but will never replace the traditional morphological evaluation.

Various compounds detected are presumably products that arise during melanisation via the 1,8-dihydroxynaphthalene pathway (Wheeler, 1983; and discussion in Stadler et al., 2001a) in the course of the maturation process. For instance, even recently collected overmature stromata of *H. bovei* (data not shown) did not yield sufficient extractable material for HPLC, in contrast to the juvenile ascigenous specimens listed in Table 3. Hence, especially the young, growing stromata of sect. *Annulata* should be analysed for additional metabolites in comparison with mature ones as already accomplished with *H. cohaerens* in this study. However promising, the results should be confirmed by examining a larger number of materials of different developmental stages to further assess the significance of secondary metabolite profiles for taxonomic purposes. Such work is currently under way.

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 MeOH. UV spectra were obtained on a Shimadzu UV-1650PC instrument in MeOH. IR spectra were measured on a Perkin–Elmer Spectrum One FT-IR spectrometer. The ^{1}H and ^{13}C NMR spectra were recorded on a Varian Unity 600 NMR spectrometer (600 MHz for ^{1}H and 150 MHz for ^{13}C), using $CDCl_3$ as solvent. Chemical shifts are given relative to TMS (δ 0.00) as internal standard (^{1}H) and δ 77.0 (ppm) from $CDCl_3$ as standard (^{13}C). Mass spectra were recorded on a JEOL JMS AX-500 spectrometer. Column chromatography was carried out on Sephadex LH-20 (Amersham Pharmacia Biotech, $CHCl_3$–MeOH, 1:1) and silica gel 60 (0.2–0.5 mm, and 0.04–0.063 mm, Merck). Preparative HPLC was carried out on a Gilson Abimed system (Mühlbauer et al., 2002).

3.2. Materials examined and methodology used for HPLC profiling

Fruiting bodies of *Hypoxylon cohaerens* Ww 4317 were used for isolation of the cohaerins. Voucher speci-

mens of this material and all other specimen materials designated JF, STMA, YMJ and Ww are deposited in the mycological herbarium of the Fuhlrott-Museum, Wuppertal, Germany (WUP) and/or in the personal herbaria of the authors. Aside from this material, several further specimens of *Hypoxylon* sect. *Annulata* were studied for comparison, using the experimental procedures previously described in detail (Mühlbauer et al., 2002; Hellwig et al., 2005) or, in case of irreplaceable old type specimens, the refined material-saving technique described by Stadler et al. (2004a). Details on these materials are compiled in Table 3. Abbreviations and addresses of herbaria that kindly provided specimens are listed in "Index Herbariorum" (Holmgren et al., 1990 and Internet database at http://www.nybg.org/bsci/ih). Previously obtained data (Stadler et al., 2001a, 2004a,b; Mühlbauer et al., 2002; Hellwig et al., 2005) on other *Hypoxylon* species were also taken into consideration and the HPLC chromatograms stored from previous work were screened for the occurrence of cohaerins A and B (**1**–**2**), using internal and external standards. For this purpose, extracts of the specimens were prepared and analysed by HPLC–UV/Vis and HPLC-MS in the positive and negative ESI mode in two different gradient systems, following the protocols and using the gradients described in Mühlbauer et al. (2002) and Stadler et al. (2004a). The old HPLC data were re-examined by manual comparison as well as by an automated search using the HPLC–UV library function of the Agilent (Waldbronn, Germany) HP Chem Station software. With the stromata of *H. cohaerens* Ww 4317, extracts were also made from granules that had been prepared under a dissecting microscope and the HPLC data compared to that of the extract made under conventional conditions from the whole stomata, showing that cohaerins are preferably located in these granules. In addition, the metabolite production by *H. cohaerens* was observed throughout the vegetation period by analysing samples prepared from stromata at different degrees of maturity (see Section 2).

3.3. Extraction of *H. cohaerens*, isolation and physicochemical properties of cohaerins

The stromata (fruiting bodies) of *H. cohaerens* Ww 4317 (11 g) were extracted with MeOH (3 × 500 ml) at room temperature in an ultrasonic bath for each 30 min, with the MeOH is whole concentrated in vacuo to give a residue (900 mg), which was subjected to reversed phase preparative HPLC using a MZ Analysentechnik (Mainz, Germany) Kromasil C18 (250 × 40 mm; 7 μm) column. The latter was eluted with a gradient of 0.1% TFA(A): CH_3CN (B) as mobile phase at 7 ml/min as follows: a linear gradient of A:B (70:30) at t = 0 min to A:B (1:1) at t = 70 min, followed by a linear gradient to 100% and at t = 100 min, this being held for 30 min. Several intermediate fractions were thus obtained, some of which could not be further purified since they contained minor or apparently unstable constituents. The main accessible metabolites were located in fractions 3 (eluting at Rt = 98–114 min), 6 (Rt = 122–123 min) and 7 (Rt = 124–127 min). Fraction 3 (138.9 mg) eluted at A:B (3:7) and was further subjected to Sephadex LH-20 CC using $CHCl_3$:MeOH (1:1) as eluent to afford BNT (**3**) (33.1 mg). Fractions 6 and 7 were next subsequently eluted with A:B (1:4), with Fraction 6 (29.7 mg) further purified by silica gel CC using $CHCl_3$:MeOH:H_2O (25:2.5:0.1) to yield cohaerin A (**1**) (9.1 mg) and cohaerin B (**2**) (12.5 mg). Fraction 7 (46.8 mg) was treated as for fraction 6 to give additional cohaerin A (**1**) (19.8 mg).

3.3.1. Cohaerin A (*1*)

Yellow oil, $[\alpha]_D^{20}$ +113.7° (*c* 0.4, $CHCl_3$). Positive FAB-MS: 459 $[M + Na]^+$; HR-FABMS *m/z* 459.1808 ($C_{26}H_{28}O_6Na$, requires *m/z* 459.1784). Positive ESI-HPLC-MS: 437 $[M + H]^+$; Negative ESI-HPLC-MS: 435 $[M - H]^-$ (Rt = 6.8 min; Fig. 3). UV λ_{max} nm ($\log \varepsilon$): 219.6 (4.2), 336.6 (3.9). CD (MeOH) λ_{ext} nm ($\Delta\varepsilon$) 359 (+1.04), 275 (−0.29), 237 (−0.20). IR (KBr): 3242, 1704, 1616, 1542, 1465, 1325, 1291, 1127, 992 cm^{-1}. ^1H and ^{13}C NMR (Table 1). HPLC–UV/Vis: Rt = 9.92 min (Fig. 3).

3.3.2. Cohaerin B (*2*)

Yellow oil, $[\alpha]_D^{20}$ +87.1° (*c* 1.0, $CHCl_3$). Positive FAB-MS: 477 $[M + Na]^+$; HR-FABMS *m/z* 477.1898 ($C_{26}H_{30}O_7Na$, requires *m/z* 477.1889). Positive ESI-HPLC-MS: 455 $[M + H]^+$; Negative ESI-HPLC-MS: 453 $[M - H]^-$ (Rt = 6.3 min, Fig. 3). UV λ_{max} nm ($\log \varepsilon$): 223.4 (4.4), 333.2 (4.2). CD (MeOH) λ_{ext} nm ($\Delta\varepsilon$) 356 (+0.61), 274 (−0.30), 226 (+0.24). IR (KBr): 3425, 1704, 1673, 1633, 1551, 1453, 1377, 1223, 972 cm^{-1}. ^1H and ^{13}C NMR (Table 1). HPLC–UV/Vis: Rt = 8.85 min (Fig. 3).

3.3.3. Acetylation of cohaerin A (*1a*)

Cohaerin **1** (8.2 mg) was acetylated using AC_2O (1 ml) in pyridine (1 ml), with work up as usual, to yield **1a** (3.2 mg) as an oil $[\alpha]_D^{20}$ +23.3° (*c* 0.3, $CHCl_3$). Positive FAB-MS: 479 $[M + H]^+$; HR-FABMS *m/z* 479.2088 ($C_{28}H_{31}O_7$, requires *m/z* 479.2070). UV λ_{max} nm ($\log \varepsilon$): 214.6 (4.3), 332.8 (4.0). IR (KBr): 1773, 1719, 1645, 1464, 1370, 1195, 996 cm^{-1}. ^1H NMR ($CDCl_3$): δ7.97 (1H, *d*,*J* = 1.4 Hz, H-1), 7.41 (1H, *t*,*J* = 7.7 Hz, H-13), 7.19 (1H, *d*,*J* = 7.7 Hz, H-12), 7.01 (1H, *d*,*J* = 7.7 Hz, H-14), 6.99 (1H, *dt*,*J* = 1.4, 7.1 Hz, H-3′), 6.32 (1H, *s*, H-4), 5.64 (1H, *d*,*J* = 1.1 Hz, H-5), 2.37 (3H, *s*, H-16), 2.26 (3H, *s*, CH_3CO-15), 2.19 (2H, *m*, H-4′), 1.86 (3H, *d*,*J* = 1.4 Hz, H-9′), 1.63 (3H, *s*, H-9), 1.46 (2H, *m*, H-5′), 1.32 (4H, *m*, H-6′ and H-7′), 0.90 (2H, *t*,*J* = 7.1 Hz, H-8′). ^{13}C NMR ($CDCl_3$): δ 193.4

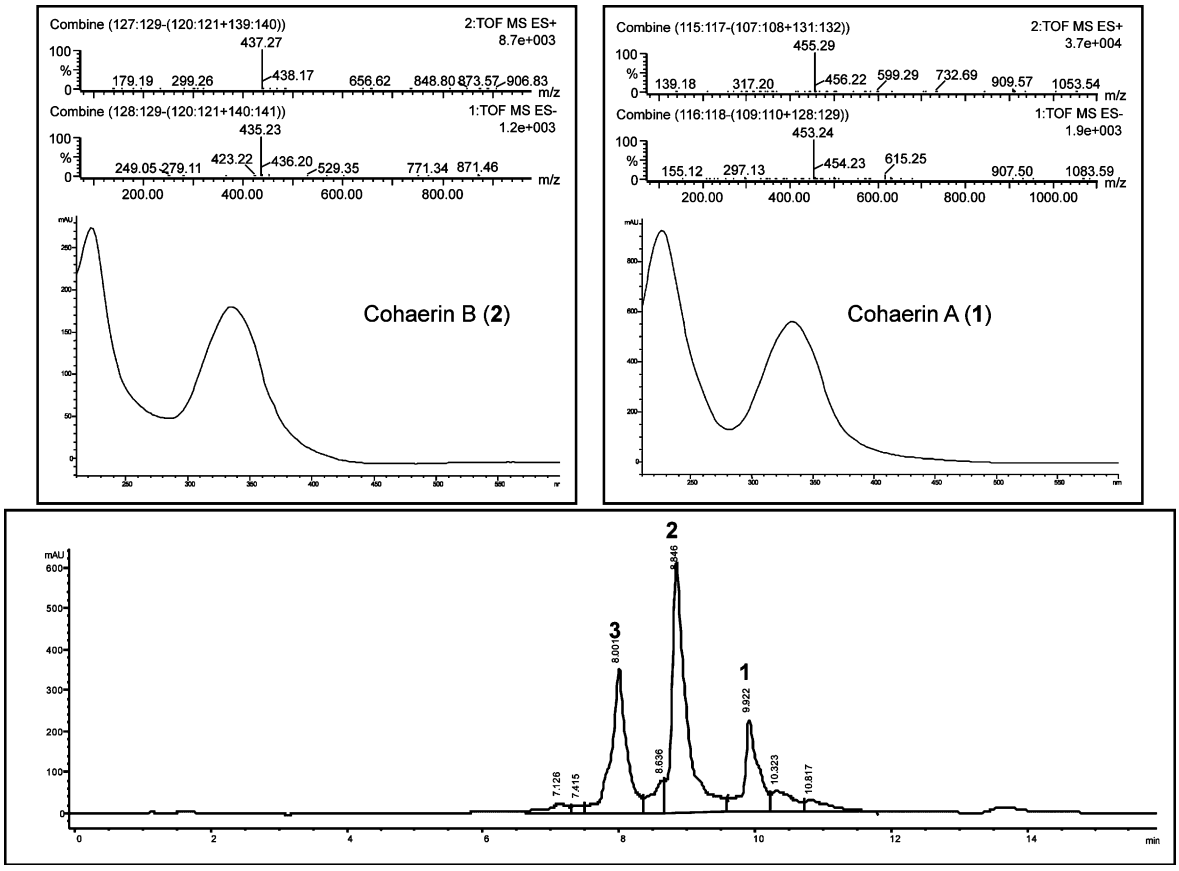

Fig. 3. HPLC–UV chromatogram of crude MeOH extract of *Hypoxylon cohaerens*, including characteristic HPLC–UV/Vis and HPLC-MS spectra of cohaerins A (**1**) and B (**2**). The spectra of BNT (**3**) are not shown. Parameters for the employed analytical HPLC systems see Mühlbauer et al. (2002).

(C-6), 193.1 (C-8), 169.4 (CH_3CO-15), 167.4 (C-1′), 154.2 (C-1), 154.1 (C-3), 149.0 (C-15), 145.7 (C-3′), 141.6 (C-4a), 139.3 (C-11), 131.4 (C-13), 128.3 (C-12), 126.0 (C-2′), 124.8 (C-10), 120.5 (C-14), 115.1 (C-8a), 113.4 (C-4), 108.3 (C-5), 84.2 (C-7), 31.6 (C-6′), 29.0 (C-4′), 28.1 (C-5′), 22.5 (C-7′), 22.1 (C-9), 20.9 (CH_3CO-15), 19.9 (C-16), 14.0 (C-8′), 12.2 (C-9′).

3.4. Other metabolites

While BNT (**3**) was isolated from *H. cohaerens*, truncatone (**5**) (7 mg) (from 100 mg of crude extract of *H. bovei* var. *microspora* YMJ 91111212) and daldinone A (**4**) (4 mg) (from 100 mg crude extract of *H. stygium* YMJ 91101906), respectively, were isolated to purity by C18 HPLC in a similar manner as for the cohaerins. Their identities were established by comparison of their NMR, MS and HPLC data with that of authentic samples.

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References

Buchanan, M.S., Hashimoto, T., Yasuda, A., Takaoka, S., Kan, Y., Asakawa, Y., 1995. The structures of novel cytochalasins, azaphilones, and aromatic compounds isolated from five ascomyceteous fungi. In: Proceedings of the 37th Symposium on the Chemistry of Natural Products, Symposium Papers, Tokushima, Japan, 1995, pp. 409–414.

Gill, M., 2003. Pigments of fungi (Macromycetes). Nat. Prod. Rep. 20, 615–639.

Gill, M., Steglich, W., 1987. Pigments of fungi (Macromycetes). In: Herz, W., Grisebach, H., Kirby, G.W., Tamm, C. (Eds.), Progress in the Chemistry of Organic Natural Products, vol. 51. Springer, Berlin, Heidelberg, New York.

Hasegawa, T., 1985. Manufacture and use of cis-3-alkenoic acid. Japanese patent, JP 60028950.

Hashimoto, T., Asakawa, Y., 1998. Biologically active substances of Japanese inedible mushrooms. Heterocycles 47, 1067–1110.

Hayashi, K., Hatsutori, N., Donho, M., Nakajima, H., 1996. Dihydrofolate reductase inhibitors containing mitorubrin compounds for protozoiasis. Japanese patent, JP 08217673.

Hellwig, V., Ju, Y.-M., Rogers, J.D., Fournier, J., Stadler, M., 2005. Hypomiltin, a new azaphilone from *Hypoxylon hypomiltum* and chemotypes in *Hypoxylon* sect. *Hypoxylon* as inferred from analytical HPLC profiling. Mycological Progress 4, 39–54.

Holmgren, P.K., Holmgren, N.H., Barnett, L.C., 1990. Index herbariorum. Part 1: Herbaria of the World, 86th ed. Botanical Gardens, Bronx, New York.

Ju, Y.-M., Rogers, J.D., 1996. A revision of the genus *Hypoxylon*. Mycologia Memoir no. 20. APS Press, St. Paul, MN., USA.

Ju, Y.-M., Rogers, J.-D., Hsieh, H.-M., 2004. New *Hypoxylon* species and notes on some names associated with or related to *Hypoxylon*. Mycologia 96, 154–161.

Koyama, K., Kuramochi, D., Kinoshita, K., Takahashi, K., 2002. Hypoxylonols A and B, novel reduced benzo[*j*]fluoranthene derivatives from the mushroom *Hypoxylon truncatum*. J. Nat. Prod. 65, 1489–1490.

Miller, J.H., 1961. A Monograph of the World Species of *Hypoxylon*. University of Georgia Press, Athens.

Mühlbauer, A., Triebel, D., Persoh, D., Wollweber, H., Seip, S., Stadler, M., 2002. Macrocarpones, novel metabolites from stromata of *Hypoxylon macrocarpum*, and new evidence on the chemotaxonomy of *Hypoxylon*. Mycol. Prog. 1, 235–248.

Quang, D.N., Hashimoto, T., Tanaka, M., Baumgartner, M., Stadler, M., Asakawa, Y., 2002. Chemical constituents of the ascomycete *Daldinia concentrica*. J. Nat. Prod. 65, 1869–1874.

Quang, D.N., Hashimoto, T., Toyota, M., Asakawa, Y., 2003. Occurrence of a high concentration of spider pheromones in the ascomycete fungus *Hypoxylon truncatum*. J. Nat. Prod. 66, 1613–1614.

Quang, D.N., Hashimoto, T., Tanaka, M., Stadler, M., Asakawa, Y., 2004a. Cyclic azaphilones daldinins E and F from the ascomycete fungus *Hypoxylon fuscum* (Xylariaceae). Phytochemistry 65, 469–473.

Quang, D.N., Hashimoto, T., Stadler, M., Asakawa, Y., 2004b. New azaphilones from the inedible mushroom *Hypoxylon rubiginosum*. J. Nat. Prod. 67, 1152–1155.

Rayner, R.W., 1970. A Mycological Colour Chart. Commonwealth Mycological Institute, Kew.

Rogers, J.D., Candoussau, F., 1980. A new variety of *Hypoxylon cohaerens* from France. Mycologia 72, 826–829.

Ruden, R.A., Litterer, W.E., 1975. Mechanism of lithium dimethylcuprate addition to conjugated enones. Tetrahedron Lett. 25, 2043–2044.

Stadler, M., Wollweber, H., Mühlbauer, A., Henkel, T., Asakawa, Y., Hashimoto, T., Rogers, J.D., Ju, Y.-M., Wetzstein, H.-G., Tichy, H.-V., 2001a. Secondary metabolite profiles, genetic fingerprints and taxonomy of *Daldinia* and allies. Mycotaxon 77, 379–429.

Stadler, M., Wollweber, H., Mühlbauer, A., Asakawa, Y., Hashimoto, T., Rogers, J.D., Ju, Y.-M., Wetzstein, H.-G., Tichy, H.-V., 2001b. Molecular chemotaxonomy of *Daldinia* and other Xylariaceae. Mycol. Res. 105, 1191–1205.

Stadler, M., Ju, Y.-M., Rogers, J.D., 2004a. Chemotaxonomy of *Entonaema*, *Rhopalostroma* and other Xylariaceae. Mycol. Res. 108, 239–256.

Stadler, M., Wollweber, H., Fournier, J., 2004b. A host-specific species of *Hypoxylon* from France, and notes on the chemotaxonomy of the *Hypoxylon rubiginosum* complex. Mycotaxon 90 (1), 187–211.

Steglich, W., Klaar, M., Furtner, W., 1974. (+)-Mitorubrin derivatives from *Hypoxylon fragiforme*. Phytochemistry 13, 2874–2875.

Steyn, P.S., Vleggaar, R., 1976. The structure of dihydrodeoxy-8-*epi*-austdiol and the absolute configuration of the azaphilones. J. Chem. Soc. Perkin Trans. 1, 204–206.

Whalley, M.A., Ju, Y.-M., Rogers, J.D., Whalley, A.J.S., 2000. New xylariaceous fungi from Malaysia. Mycotaxon 74 (1), 135–140.

Wheeler, M., 1983. Comparisons of fungal melanin biosynthesis in ascomyceteous, imperfect and basidiomyceteous, fungi. Trans. Brit. Mycol. Soc. 81, 29–36.

Yoon, C.-S., Glawe, D.A., 1993. Association of random amplified polymorphic DNA markers with stromatal type in *Hypoxylon truncatum* sensu Miller. Mycologia 85, 369–380.