Carneic Acids A and B, Chemotaxonomically Significant Antimicrobial Agents from the Xylariaceous Ascomycete *Hypoxylon carneum*

Dang Ngoc Quang,[†,⊥] Marc Stadler,*[,§] Jacques Fournier,[‡] and Yoshinori Asakawa[†]

Faculty of Pharmaceutical Sciences, Tokushima Bunri University, Yamashiro-cho, Tokushima 770-8514, Japan, InterMed Discovery GmbH, BioMedizinZentrum Dortmund, Otto-Hahn-Strasse 15, D-44227 Dortmund, Germany, Las Muros, F-09420 Rimont, Ariége, France, and Faculty of Chemistry, Hanoi University of Education, 136 Xuan Thuy Road, Cau Giay, Hanoi, Vietnam

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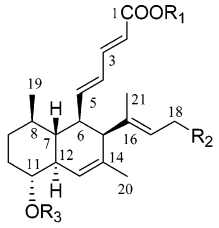
Carneic acids A and B (**1**, **2**) are polyketide antibiotics structurally related to phomopsidin. They were isolated as major constituents of the stromata of *Hypoxylon carneum*, a species that had shown a highly specific secondary metabolite profile in a survey of xylariaceous ascomycetes based on HPLC profiling. Their chemical structures were elucidated by a combination of spectroscopic methods and by preparation of derivatives. An X-ray crystal structure of the dinitrobenzoate of carneic acid B methyl ester (**8**) was obtained, even allowing for determination of its absolute structure. The carneic acids showed weak antibacterial and moderate antifungal activities in the serial dilution assay against selected microbial organisms. They appear to be species-specific marker molecules in *H. carneum* from different geographic regions, but do not constitute major metabolites of more than 100 species of Xylariaceae. Their biological and chemotaxonomic significance is discussed.

Hypoxylon carneum Petch (Xylariaceae, Figure 1) is a wood-inhabiting ascomycete, first described from Sri Lanka[1] and later also reported from the United States, Venezuela, New Zealand,[2] and France.[3] It is one of the few species in the large genus *Hypoxylon* that is not restricted to certain geographic areas, but was proven to have a truly cosmopolitan distribution, being present in temperate as well as in subtropical and tropical climates. As judged from our field studies on its occurrence in southwestern France,[3] it is apparently not rare. Because of its morphological similarity to the common *H. rubiginosum* and other related species, it has probably been frequently overlooked.[3] The most striking feature to discriminate *H. carneum* from its relatives can be readily determined without any need for detailed microscopic studies: its pigments in KOH are faint purple, while those of related species yield dense orange, brown, or yellowish pigments in KOH.[3] Also, *H. carneum* has faintly yellowish granules beneath the stromata surface, whereas those of its allies are orange, red, or brown. These features are due to the presence of different secondary metabolites, which was established by a HPLC profiling study of about 2500 specimens and cultures of *Hypoxylon* and related Xylariaceae.[3−6] Recent evidence for the molecular taxonomy of *Hypoxylon* and allies[7] revealed that the closest affinities of *H. carneum* (i.e., the clades H1 and H2 as described in ref 7), as inferred from a molecular phylogenetic study, are with species that preferably contain azaphilones. These clades comprise, aside from some taxa that were not yet studied for HPLC profiles, for example, *H. cercidicola*, *H. petriniae*,[3] *H. perforatum*, *H. pilgerianum*, and *H. rubiginosum*.[3,4] *H. carneum* strongly differed from all of these species in its HPLC profile. The common stromatal azaphilone pigments of many *Hypoxylon* spp. of clades H1 and H2 as described in ref 7 (i.e., mitorubrins, rubiginosins, and hypomiltin[3,4]) were not detected by HPLC-MS, and instead 4:5:4′:5′-tetrahydroxy-1:1′-binaphthyl (BNT, **3**) and a series of apparently species-specific but yet unknown metabolites were detected as major components.[4] Interestingly, only BNT (**3**) and daldinins, but no mitorubrin-type azaphilones, were also reported previously from the core species of another side branch of clade H1, i.e., *H. fuscum*.

We have recently checked the extracts of various *Hypoxylon* spp. derived from the above studies for antimicrobial activities. The stromatal MeOH extract of *H. carneum* showed significantly stronger antifungal activities than those prepared from its azaphilone-containing relatives (Table S1, Supporting Information). Recently, the fungus was collected in sufficient amounts for preparative studies. We here report the identification of **1** and **2** and preliminary studies on their biological properties.



1. R_1 = H; R_2 = H; R_3 = H

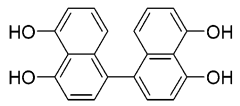
2. R_1 = H; R_2 = OH; R_3 = H

5. R_1 = Me; R_2 = H; R_3 = H

6. R_1 = Me; R_2 = OH; R_3 = H

7. R_1 = Me; R_2 = H; R_3 = *p*-NO_2-C_6H_4-CO-

8. R_1 = Me; R_2 = R_3 = *p*-NO_2-C_6H_4-COO-



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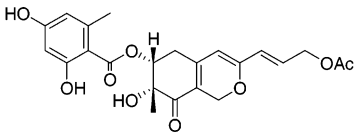

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Results and Discussion

Carneic acids A and B (**1**, **2**) were detected in and isolated from the stromatal methanol extract of *H. carneum* as described in the Experimental Section, along with BNT (**3**).[6] In addition, rubiginosin A (**4**) and orsellinic acid (i.e., characteristic major metabolites of many *Hypoxylon* spp.[3,8]) were detected by HPLC profiling[4] in the fractions derived from preparative chromatography after removal of the major components (**1**−**3**). The presence of these compounds

* Corresponding author. E-mail: marc.stadler@t-online.de.
† Tokushima Bunri University.
§ InterMed Discovery GmbH.
‡ Las Muros.
⊥ Hanoi University of Education.

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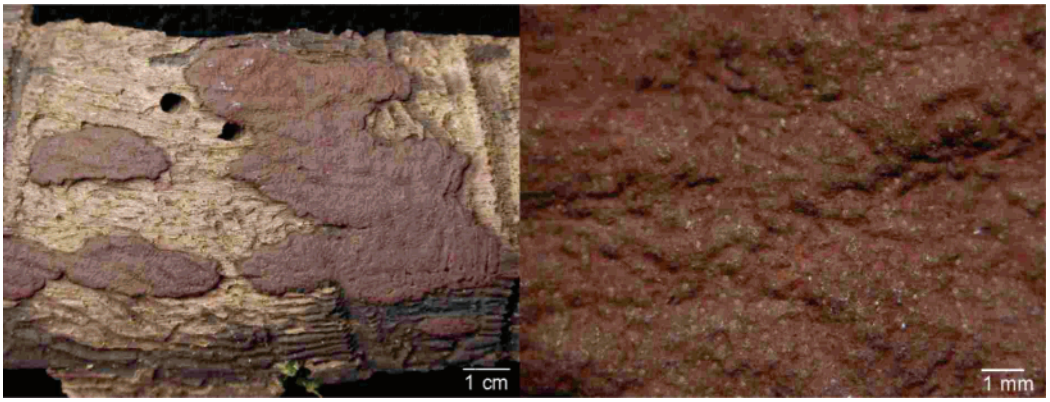

Figure 1. Stromata of *Hypoxylon carneum*. Left: Habit on wood. Right: Augmented section, showing perithecial mounds. Scale is indicated by bars.

Table 1. ^{1}H NMR Data for **1**, **2**, and **5**−**8**

position	**1**	**2**	**5**	**6**	**7**	**8**
2	5.79 (d, 15.1)	5.79 (d, 15.1)	5.79 (d, 15.3)	5.81 (d, 15.1)	5.82 (d, 15.4)	5.83 (d, 15.4)
3	7.26 (dd,10.1, 15.1)	7.27 (dd, 9.9, 15.4)	7.29 (dd, 10.1, 15.3)	7.28 (dd, 10.7, 15.4)	7.30 (dd, 10.4, 15.3)	7.26 (dd, 11.0, 15.4)
4	6.12 (overlapped)	6.20 (dd, 10.2, 15.1)	6.09 (dd, 10.2, 15.1)	6.12 (dd, 10.3, 15.1)	6.13 (dd, 10.2, 14.8)	6.15 (dd, 11.0, 15.1)
5	6.12 (overlapped)	6.16 (dd, 10.0, 15.1)	6.06 (dd, 9.9, 15.1)	6.05 (dd, 10.2, 15.1)	6.07 (dd, 9.6, 15.1)	6.04 (dd, 10.4, 15.2)
6	2.46 (m)	2.51 (dd, 6.0, 10.2)	2.45 (m)	2.50 (dt, 6.0, 17.0)	2.49 (dd, 6.0, 10.4)	2.58 (dt, 6.3, 10.7)
7	1.29 (t, 9.9)	1.34 (m)	1.28 (t, 9.9)	1.29 (m)	1.44 (t, 9.9)	1.44 (q, 10.7)
8	1.25 (m)	1.26 (m)	1.25 (m)	1.27 (m)	1.34 (m)	1.35 (m)
9a	1.14 (m)	1.16 (dt, 3.6, 13.5)	1.13 (m)	1.14 (m)	1.26 (m)	1.25 (m)
9e	1.68 (m)	1.68 (m)	1.67 (m)	1.67 (dd, 3.6, 13.7)	1.76 (m)	1.76 (m)
10a	1.37 (m)	1.36 (m)	1.36 (m)	1.37 (m)	1.54 (m)	1.56 (m)
10e	1.99 (dd, 3.9, 12.4)	1.94 (quin, 4.1)	1.98 (m)	1.99 (m)	2.18 (m)	2.19 (m)
11	3.27 (dt, 4.1, 10.4)	3.71 (dt, 4.4, 10.4)	3.26 (dt, 4.4, 10.4)	3.27 (dt, 4.4, 10.4)	4.77 (dt, 4.7, 10.7)	4.79 (dt, 4.7, 10.9)
12	1.70 (m)	1.71 (m)	1.70 (m)	1.72 (m)	2.18 (m)	2.20 (m)
13	5.83 (brs)	5.91 (brs)	5.83 (brd, 1.4)	5.88 (brd, 1.4)	5.46 (brs)	5.53 (brd, 1.1)
15	2.51 (d, 5.5)	2.55 (m)	2.50 (d, 5.8)	2.54 (d, 6.0)	2.53 (d, 7.4)	2.64 (d, 5.5)
17	5.22 (dd, 1.4, 6.8)	5.39 (dt, 1.1, 6.6)	5.21 (dd, 1.1, 6.8)	5.41 (dt, 1.1, 6.6)	5.23 (dd, 1.4, 6.8)	5.47 (dt, 1.1, 5.8)
18	1.61 (d, 6.5)	4.17 (dd, 6.6, 13.8) 4.12 (dd, 6.3, 13.8)	1.61 (d, 6.8)	4.23 (dd, 6.9, 12.9) 4.20 (dd, 6.3, 12.9)	1.62 (d, 6.5)	4.92 (d, 6.9)
19	0.86 (d, 5.8)	0.90 (d, 6.0)	0.86 (d, 5.8)	0.86 (d, 6.0)	0.91 (d, 6.3)	0.90 (d, 6.0)
20	1.54 (s)	1.57 (s)	1.54 (d, 1.4)	1.57 (s)	1.48 (s)	1.51 (s)
21	1.57 (s)	1.66 (s)	1.57 (s)	1.65 (s)	1.58 (s)	1.78 (s)
1-OMe			3.74 (s)	3.74 (s)	3.75 (s)	3.74 (s)
benzoate					8.13 (d, 9.1)	8.32 (d, 9.1)
					8.25 (d, 9.1)	8.25 (d, 9.1)
						8.21 (d, 9.1)

in *H. carneum* is in accordance with the taxonomic relationships between the *H. rubiginosum* complex and *H. carneum* that had previously been established on the basis of morphological and molecular data.[3,4,7] However, **1** and **2** were not detected in any of the fractions still available from previous work on *H. rubiginosum* and allied taxa containing mitorubrin-type azaphilones,[8] by the established HPLC profiling methodology. Retrospective HPLC-MS analysis of the data on all specimens of *Xylariaceae* examined previously,[3−5] using **1** and **2** as external standards, also did not give any indication as to their presence in over 20 additional species. However, **1** and **2** were also revealed to be major components of the holotype material of *H. carneum* and other specimens. Therefore, the taxonomic significance of carneic acids appears to be restricted to the species level.

Extraction of the stromatal surface of *H. carneum* specimens after careful detachment from the perithecial layer as described previously[4] revealed larger amounts of BNT (**3**) in relation to the carneic acids (**1**, **2**) in the resulting MeOH extracts by HPLC. Therefore, the new compounds are presumably concentrated in the granules embedded in waxy layers, surrounding the perithecia, while (as in other *Hypoxylon* spp.[4]) the binaphthyl **3** is preferentially located at the surface. The fact that **1** and **2** are not exposed to air or light might be the reason these compounds proved stable in old herbarium specimens for decades. It even gives some indication of their possible biological functions as hidden "defense weapons" of *H. carneum* that are only exposed if the fruiting bodies are attacked by feeding enemies

Carneic acid A (**1**) was isolated as an oil. It was formulated as $C_{21}H_{30}O_3$ by HREIMS. Its IR and UV spectra exhibited absorption bands due to a conjugated carboxylic group (3500−2300, 1687 cm^{-1} and 267 nm, respectively) and double bonds (1634 cm^{-1}). The ^{1}H NMR spectrum of **1** (Table 1) revealed the presence of six olefinic protons, one tertiary methyl, three vinylic methyls, and two methylenes. The ^{1}H−^{1}H COSY spectra of **1** showed connectivities from C-2 to C-13, between C-6 and C-15, and between C-17 and C-18. Careful analysis of its HMQC and HMBC spectra suggested that **2** contained a pentadienoic acid and 1-methylpropenyl partial structures, which connected with the decaline skeleton at C-6 and C-15, respectively, on the basis of HMBC correlations between H-5 and C-6 and between H-15 and C-17. The absolute structure of **2** was established by CD and NOESY spectra. Two double bonds of the pentadienoic acid unit and the double bond C_{16-17} were all

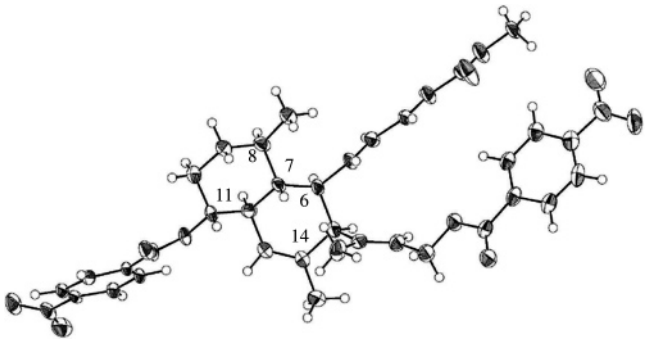

Figure 2. ORTEP drawing of **8**.

determined to be *E*-form from their coupling constants (Table 1) and NOESY correlation between H-15 and H-17. Four protons, H-6, H-8, H-10a, and H-12, were all axial and α-face, due to NOESY correlations between H-6/H-8 and H-12, H-8/H10a and H-12, and H10a/H-12; therefore, the methyl group CH_3-19 was equatorial. Furthermore, H-7, H-9a, and H-11 were also axial, but *β*-face on the basis of the NOESY correlations between H-7/H-9a and H-11, H-9a, and H-11. In addition, H-17 correlated with H-7 in the NOESY spectrum, indicating that H-15 was equatorial. This was confirmed by the coupling constant of H-15 (d, 5.5 Hz), suggesting that the dihedral angle of H-15 and H-6 was estimated to be about 60°. Meanwhile, H-13 appeared as a broad singlet because the dihedral angle between H-13 and H-12 was about 90°.[9] To determine the absolute structure of **1**, it was methylated to obtain **5**, following by *p*-nitrobenzoylation to afford **7**. The CD spectrum of **7** showed positive (283 nm) and negative (243 nm) Cotton effects, indicating the *R*-configuration at C-11.[10] From the above data, carneic acid A (**1**) was found to be 5-[5*R*-hydroxy-3,8*R*-dimethyl-2*S*-(1-methylpropenyl)-1*R*,2*S*,4a*R*,5,6,7,8*R*,8a*R*-octahydronaphthalen-1-yl]penta-2*E*,4*E*-dienoic acid.

Carneic acid B (**2**) was also obtained as an oil. It has the molecular formula $C_{21}H_{30}O_4$ based on HREIMS with one more oxygen atom than **1**. Its spectroscopic data were identical with those of **1** except for the presence of a 3-hydroxy-1-methylpropenyl unit in the place of the 1-methylpropenyl partial structure. To establish its absolute structure, compound **2** was methylated by $(CH_3)_3SiN_2$-CH in methanol to give **6**, then benzoylated by *p*-nitrobenzoyl chloride to afford **8** as white crystals. Taking into account its ORTEP drawing (Figure 2) and NOESY and CD spectra allowed us to establish its absolute structure as 5-[5*R*-hydroxy-2*S*-(3-hydroxy-1-methylpropenyl)-3,8-dimethyl-1*R*,2*S*,4a*R*,5,6,7,8*R*,8a*R*-octahydronaphthalen-1-yl]penta-2*E*,4*E*-dienoic acid.

Crude extracts of various *Hypoxylon* spp. that are known to contain azaphilones,[3] as well the pure compounds **1**−**4**, were tested for biological activity in the serial dilution assay against several filamentous fungi, a Gram-positive bacterium, and one yeast (Table 3 and Table S1, Supporting Information). Carneic acids (**1**, **2**), as well as the extract of their producer organism, showed somewhat enhanced activity against filamentous fungi and the yeast *Yarrowia lipolytica*. *Bacillus subtilis* was about equally affected by rubiginosin A (**4**), BNT (**3**), and the crude extracts of the other *Hypoylon* spp. studied. The stromata of all of those fungi contain azaphilones and/or BNT (**3**) as major metabolites,[3,4] but, according to the results of our HPLC profiling study, they are apparently devoid of compounds **1** and **2**. The results, along with the extraordinary amounts of carneic acids present in the stromata of *H. carneum* (isolated yields ca. 4−5% of **1** and **2** combined, not accounting for losses during chromatography), indicate that this species may have developed a specific chemical defense system that is absent in its closest relatives and may be directed toward fungi.

The closest structural analogue of the carneic acids (**1**, **2**) is phomopsidin, an antibiotic from a marine-derived *Phomopsis* sp. that was shown to inhibit microtubule assembly.[9,10] Additional

Table 2. ^{13}C NMR Data for **1**, **2**, and **5**−**8**

position	**1**	**2**	**5**	**6**	**7**	**8**
1	172.4	170.8	167.9	167.8	167.8	167.6
2	118.0	120.4	118.4	118.9	118.8	119.4
3	147.3	146.8	145.3	144.9	145.0	144.5
4	125.8	127.5	125.8	126.3	126.2	126.8
5	152.4	151.9	151.1	150.1	150.2	148.8
6	50.3	51.6	50.2	49.9	50.3	49.8
7	43.2	44.5	43.2	43.0	43.2	43.3
8	35.5	36.8	35.5	35.4	35.2	35.2
9	35.0	36.5	35.0	35.0	34.5	34.4
10	35.8	36.6	35.9	35.9	31.7	31.7
11	73.5	74.0	73.5	73.3	77.9	77.6
12	49.9	51.0	50.2	49.9	46.6	46.4
13	122.6	125.0	122.6	123.4	121.4	122.6
14	135.2	135.2	135.3	134.3	136.4	135.1
15	56.2	57.5	56.3	56.0	56.1	56.0
16	133.6	137.4	133.7	137.2	133.4	140.9
17	124.4	130.9	124.3	129.3	124.7	123.8
18	13.6	59.6	13.6	59.5	13.7	62.5
19	22.8	23.2	22.8	22.8	22.7	22.7
20	21.7	22.1	21.7	21.7	21.7	21.7
21	17.6	18.7	17.7	18.4	17.8	18.4
1-OMe			51.5	51.5	51.1	51.6
benzoates					164.4, 150.5, 136.0, 130.7, 123.6	164.6, 164.5, 150.6, 135.9, 135.1, 130.7, 123.7, 123.6

Table 3. Comparison of Biological Activities of **1**−**4** with Those of Standard Antibiotics in the Serial Dilution Assay against *B. subtilis* (BS, NB medium, 18 h) and Various Fungi (YMG medium, 24 h)[a]

	MIC [μg mL^{-1}]					
sample	BS	YL	MH	PG	SC	TH
carneic acid A (**1**)	50	25	12.5	12.	25	50
carneic acid B (**2**)	50	25	12.5	25	25	50
BNT (**3**)	100	>100	100	>100	>100	>100
rubiginosin A (**4**)	50	100	25	>100	100	>100
penicillin G	3.12	>100	>100	>100	>100	>100
actinomycin D	>100	6.25	3.12	3.12	3.12	6.25

[a] YL, *Yarrowia lipolytica*; MH, *Mucor hiemalis*; PG, *Penicillium griseofulvum*; SC, *Stachybotrys chartarum*; TH, *Trichoderma harzianum*. Concentrations tested: 100, 50, 25, 12.5, 6.25, 3.1, 1, and 0.1 μg mL^{-1}. MIC = minimal inhibitory concentration.

structurally related compounds are the fusarielins from *Fusarium*[11] and MK8383 from a *Phoma* species.[9] Neither of these genera belong to the order Xylariales or the family Xylariaceae, in which *H. carneum* is included, and no structurally similar polyketides have been reported from any member of the latter fungal taxa, according to a recent literature survey.[12] Phomopsidin was proved to be biogenetically derived from the polyketide pathway by radiolabeling experiments.[10] A similar biogenesis of (**1**, **2**) appears plausible, even though they have a different stereochemistry and absolute configuration than phomopsidin and the other aforementioned analogues. Since biogenetic pathways are usually stereospecific, *H. carneum* may have evolved an analogous polyketide synthesis.

Despite being apparently more specific than those of the major metabolites of other Xylariaceae, the antifungal activities of the carneic acids (**1**, **2**) can still be regarded as moderate at best. It remains unclear as to whether their mode of action is the same as that of phomopsidin and MK8383 (i.e., the cytoskeleton[9]). In previous work, the stereochemistry of the decaline moiety, as well as the presence of a free carboxyl function in the pentadienic acid side chain, was considered to be essential for activity of this class of fungal metabolites in the microtubule assembly assay.[9,10] In the same study, it was postulated that fusarielin A did not show such activity since it differed in both aforementioned features, but still that compound was shown to have antifungal effects.[11] The carneic

acids possess a free pentadienic acid moiety, but differ from phomopsidin in the stereochemistry of the decaline skeleton.

We reported previously[4] that there are further *Hypoxylon* spp., aside *from H. carneum*, that are devoid of azaphilone pigments and produce apparently specific but yet unidentified compounds instead. The outcome of the current project suggests that it may be rewarding to focus further on such fungal taxa in a search for further new bioactive chemical entities. However, location and identification of these rare species need considerable mycological expertise. Thus, close interdisciplinary collaborations, as well as the endowment of chemotaxonomy in bioprospecting, will increase the chances to discover further untapped biological sources and novel lead structures.[13]

Experimental Section

General Experimental Procedures. Melting points were determined on a Yanagimoto micromelting point apparatus and are uncorrected. Optical rotations were measured on a JASCO DIP-1000 polarimeter with $CHCl_3$ as solvent. UV spectra were obtained on a Shimadzu UV-1650PC instrument in MeOH. CD spectra were measured on a JASCO J-725 spectrometer in EtOH. IR spectra were measured on a 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 (δ 0.00) as internal standard (^1H) and δ 77.0 (ppm) from $CDCl_3$ and δ 49.0 (ppm) from CD_3OD as standards (^{13}C). Mass spectra including high-resolution mass spectra were recorded on a JEOL JMS AX-500 spectrometer. X-ray reflection data were measured on a Bruker APEXII CCD area detector using Mo Kα radiation (λ = 0.71073 Å). Column chromatography was carried out on silica gel 60 (0.2−0.5 mm, 0.04−0.063 mm, Merck) and Sephadex LH-20 (Amersham Pharmacia Biotech). Analytical HPLC was carried out as reported previously,[3,4] using a dual system to identify characteristic components by HPLC with diode array detection and mass spectrometric detection in the positive and negative electrospray modes.

Fungal Material. Stromata of *H. carneum* Petch were collected and identified by J.F. in the vicinity of Rimont, Department of Ariége, France, on wood and bark of *Fraxinus excelsior* on April 14, 2005, and in the same locality on *Salix* sp. on November 3, 2005, respectively. A voucher specimen is deposited at the Botanische Staatsammlung, Munich, Germany (designation number JF-05048). For comparison, the original holotype material of *H. carneum* (Kew, collected in 1908 from Sri Lanka),[1,2] and specimens PDD 16333, PDD 59111, and PDD 62056 (collected during 1949−1991 from New Zealand)[2] were also studied by analytical HPLC. In addition, previously recorded HPLC profiles of crude extracts and preparative fractions (spectra and chromatograms stored in a HPLC library) were used for comparison with that of *H. carneum* and compounds **1** and **2**. Aliquots of crude MeOH extracts of related azaphilone-containing *Hypoxylon* spp. (the latter mostly deposited at the mycological herbarium of the Fuhlrott-Museum, Wuppertal, Germany) were used for comparison of biological activities. The results are compiled in Table S1 (Supporting Information).

Stromata of *H. carneum* were detached from the substrate, air-dried, and extracted with MeOH. The methanolic extract (781 mg) from 12 g of dried fungal material was chromatographed on SiO_2 using $CHCl_3$−MeOH−H_2O (25:2.5:0.1, 400 mL) to give four fractions. Fraction 3 (365 mg) was subjected to Sephadex LH-20 column chromatography ($CHCl_3$−MeOH, 1:1, 400 mL) to give carneic acid A (**1**) (304 mg) and BNT (**3**) (25.7 mg). Fraction 4 (126 mg) was pure carneic acid B (**2**). The amounts of rubiginosin A (**4**) and orsellinic acid[3] in the crude extract of *H. carneum* were estimated to be less than 0.5% each, as judged from quantitative HPLC-UV analysis using external standards. Those compounds were not isolated to purity.

Carneic acid A (1): colorless, amorphous solid; $[\alpha]^{20}_D$ −4.0 (*c* 0.5, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 267 (4.4); IR (KBr) ν_{max} 3500−2300, 1687, 1634, 1615, 1276, 1007, 758 cm^{-1}; ^1H and ^{13}C NMR spectra ($CDCl_3$), Tables 1 and 2; EIMS *m*/*z* 330 (24) $[M]^+$, 312 (26), 297 (11), 132 (25), 130 (28), 124 (32), 115 (100), 81 (39), 55 (28); HREIMS *m*/*z* 330.2190 (calcd for $C_{21}H_{30}O_3$, 330.2195).

Carneic acid B (2): colorless, amorphous solid; $[\alpha]^{20}_D$ −1.3 (*c* 1.0, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 266 (4.5); IR (KBr) ν_{max} 3500−2300, 1692, 1634, 1273, 1006, 882 cm^{-1}; ^1H and ^{13}C NMR spectra (CD_3OD), Tables 1 and 2; EIMS *m*/*z* 346 (5) $[M]^+$, 328 (19), 297 (13), 213 (11), 205 (20), 187 (81), 157 (22), 131 (30), 121 (43), 107 (62), 81 (22), 55 (27), 44 (100); HREIMS *m*/*z* 346.2148 (calcd for $C_{21}H_{30}O_4$, 346.2144).

Methylation of 1. Carneic acid A (**1**) (14 mg) was methylated with $(CH_3)_3SiN_2CH$ (1.5 mL) in MeOH (1 mL) at 5 °C for 3 h. The reaction mixture was purified by silica gel column chromatography, hexane−EtOAc (4:1), to give carneic acid A methyl ester **5** (11.6 mg): ^1H and ^{13}C NMR spectra ($CDCl_3$), Tables 1 and 2; EIMS *m*/*z* 344 (20) $[M]^+$, 245 (22), 227 (26), 205 (27), 187 (100), 159 (34), 147 (30), 133 (35), 121 (88), 107 (52), 81 (22), 55 (22); HREIMS *m*/*z* 344.2351 (calcd for $C_{22}H_{32}O_3$, 344.2351).

Methylation of 2. Carneic acid B (**2**) (20 mg) was methylated with $(CH_3)_3SiN_2CH$ (1.5 mL) in MeOH (1 mL) at 5 °C for 3 h. The reaction mixture was purified by silica gel column chromatography, hexane−EtOAc (1:1), to give carneic acid B methyl ester (**6**) (13.3 mg): ^1H and ^{13}C NMR spectra ($CDCl_3$), Tables 1 and 2; EIMS *m*/*z* 360 (3) $[M]^+$, 342 (24), 324 (16), 243 (19), 225 (19), 205 (39), 187 (100), 159 (50), 145 (52), 133 (43), 121 (55), 105 (57), 91 (59), 55 (25); HREIMS *m*/*z* 360.2301 (calcd for $C_{22}H_{32}O_4$, 360.2301).

***p*-Nitrobenzoate of 5.** To a solution of compound **5** (11.6 mg) in pyridine (1 mL) were added *p*-nitrobenzoyl chloride (20 mg) and DMAP (1 mg). The reactants were stirred overnight at room temperature and concentrated to give a residue, which was separated by silica gel column chromatography, hexane−EtOAc (4:1, 150 mL), to obtain **7** (7.3 mg): UV (MeOH) λ_{max} (log ϵ) 267 (4.3); CD (EtOH) λ_{ext} nm ($\Delta\epsilon$) 283 (−11.7), 243 (+ 11.2); ^1H and ^{13}C NMR spectra ($CDCl_3$), Tables 1 and 2; EIMS *m*/*z* 493 $[M]^+$, 327 (18), 227 (65), 187 (100), 159 (80), 148 (47), 120 (24), 105 (25), 91 (18); HREIMS *m*/*z* 493.2466 (calcd for $C_{29}H_{35}O_6N$, 493.2464).

Di-*p*-nitrobenzoates of 6. *p*-Nitrobenzoyl chloride (35 mg) and DMAP (1.2 mg) were added to a solution of **6** (13.3 mg) in pyridine. The reaction was performed in the same manner as described above and led to a mixture, which was further purified by silica gel column chromatography, hexane−EtOAc (1:1, 120 mL), to give **8** (15.6 mg) as colorless needles ($CHCl_3$): mp 160−165 °C; UV (MeOH) λ_{max} (log ϵ) 264 (4.5); CD (EtOH) λ_{ext} nm ($\Delta\epsilon$) 279 (−6.4), 246 (+ 2.9); FABMS *m*/*z* 659 $[M + H]^+$; HRFABMS *m*/*z* 659.2579 (cald for $C_{36}H_{39}O_{10}N_2$, 659.2605); ^1H and ^{13}C NMR spectra ($CDCl_3$), Tables 1 and 2.

Crystal Data for 8.[14] Data collection: Bruker APEX2. Cell refinement: Bruker APEX2. Data reduction: Bruker SAINT. Computing structure refinement: SHELXL-97.[15] Refinement: Full-matrix least-squares on F^2. $C_{36}H_{38}O_{10}N_2$ approximate dimensions 0.16 × 0.09 × 0.04 mm^3, MW 658.704, triclinic, *P*1, a = 6.564(2) Å, b = 15.654(6) Å, c = 16.701(6) Å, α = 94.618(5)°, β = 92.568(5)°, γ = 100.754(5)°, V = 1677.3(10) Å^3, Z = 2, μ = 0.096 mm^{-1}, 6697 reflections, 866 parameters; R = 0.0869, R_w = 0.2313, S = 1.027.

The biological activities were determined in a conventional serial dilution assay against various fungi and bacteria, using 48-well Nunclon microtiter plates (Nunc, Wiesbaden, Germany). Aside from *Yarrowia lipolytica* HT20 (IMD culture collection, Wuppertal/Dortmund), all test strains were obtained from public collections (ATCC, American Type Culture Collection, Manassas, VA; and DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). Overnight cultures in YMG (*Y. lipolytica*) or Difco nutrient broth (*Bacillus subtilis* ATCC 6633) served for preparation of inoculum for nonfilamentous organisms. For testing of filamentous fungi (*Mucor hiemalis* DSM 63298, *Penicillium griseofulvum* DSM 847, *Stachybotrys chartarum* DSM 2144, and *Trichoderma harzianum* ATCC 64870), spore suspensions were obtained by rinsing the surface of well-grown 9-day-old cultures propagated in Fernbach YMG agar flasks with YMG agar containing 0.1% Tween 80. Test compounds were dissolved and diluted in MeOH. Aliquots of these solutions were supplied to the wells in adequate amounts to reach the desired final concentrations (100 μg per mL and dilutions thereof; see Table 3). The solvent was evaporated in vacuo; then cell or spore suspensions obtained from overnight cultures of the test organisms were adjusted to 10^5 cells per mL and aliquots thereof incubated with MeOH solutions of the test compounds for 18 h (*B. subtilis*) or 24 h (filamentous fungi and *Y. lipolytica*), respectively.

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Supporting Information Available: Table S1, summarizing the antimicrobial activities of crude extracts of various *Hypoxylon* species. Table S2, containing a list of species so far examined in the course of our polythetic taxonomic study, using HPLC profiling, morphological, ultrastructural, and molecular data. Additional references are given where those data were previously published. In addition, a CIF file containing the X-ray data of compound **8** (corresponding to Figure 2) is provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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(14) Crystallographic data for the structure reported in this paper have been deposited at the Cambridge Crystallographic Data Centre (deposition number CCDC 604567). Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44-(0) 1223-336033 or e-mail: deposit@ccdc.cam.ac.uk).
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