

HISPOLON, A YELLOW PIGMENT FROM *INONOTUS HISPIDUS*

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Key Word Index—*Inonotus hispidus*; Basidiomycetes; mushroom pigments; hispidin; hispolon.**Abstract**—Ethanol extracts of fruit bodies from *Inonotus hispidus* showed interesting immunomodulatory and antiviral activity. Bioactivity-guided fractionation and purification afforded two phenolic compounds which were identified by their physical and spectral data as the known hispidin and a new metabolite named hispolon.

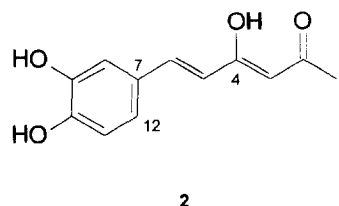
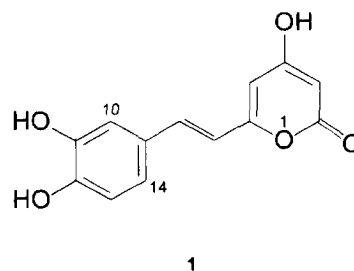
INTRODUCTION

Inonotus hispidus (Bull. ex Fr.) Karst. (Basidiomycetes) is a parasitic fungus preferably living on a variety of deciduous trees like *Fraxinus*, *Quercus*, *Sorbus* and *Malus* [1]. It produces a considerable quantity of yellow-brown pigments, e.g. hispidin (1), bis-noryangonin and hypholomin B [2-5]. Unlike *I. obliquus*, the well known Chaga fungus, *I. hispidus* is not used in folk medicine, although it is known that 1 and bis-noryangonin exhibit antimicrobial activity [6]. In our screening for immunomodulatory and antiviral agents from Basidiomycetes, inhibitory activity was detected in the ethanol extracts of fruit bodies of *I. hispidus*. Bioactivity-guided fractionation of the extract resulted in the isolation of 1 and a new pigment named hispolon (2). The present paper describes the isolation and structural elucidation of the new compound. Details of the pharmacological studies will be reported elsewhere.

RESULTS AND DISCUSSION

Compound 1 was isolated by gel chromatography on Sephadex LH-20 and crystallized from methanol. Infra-red, mass and ^1H NMR spectra were in full agreement with the reported data for 1 [2, 7]. The structure of 1 was unambiguously confirmed by the ^{13}C NMR data, which were assigned by direct ^1H , ^{13}C NMR spectroscopy as well as by comparison with the data for 2. Compound 1 was obtained as crystals in a yield of 0.78% dry weight (780 mg).

Compound 2 was isolated by column chromatography on silica gel from an ethyl acetate extract, and further enriched by repeated gel chromatography of selected fractions on Sephadex LH-20. It was finally purified by preparative TLC, yielding 0.06% dry weight (60 mg).



Since the pigment could also be identified by analytical TLC and HPLC in the extracts of fresh fruit bodies, an artefact could be excluded. Compound 2 shows a retention time of 2.7 min in analytical reversed phase HPLC.

In analytical TLC on Silica gel 60 plates with solvent B it migrates to R_f 0.56. The yellow spot of 2 can be stained with dinitrophenylhydrazine reagent, resulting in an orange colour which indicates the presence of a carbonyl group, while spraying with anisaldehyde/sulphuric acid reagent furnishes a violet colour. Due to its UV absorption maxima at 258 and 372 nm, 2 can be further visualized under UV light at 254 and 366 nm, with the latter showing a brown fluorescence.

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Table 1. ^1H and ^{13}C NMR data for hispolon in acetone- d_6 (400 and 100 MHz)

C	δ_{H}	<i>m</i>	<i>J</i>	δ_{C}	<i>m</i>
1	2.10	<i>s</i>	—	26.59	<i>q</i>
2	—	—	—	197.64	<i>s</i>
3	5.77	<i>s</i>	—	101.08	<i>d</i>
4	—	—	—	179.00	<i>s</i> *
5	6.46	<i>d</i>	15.8	120.56	<i>d</i>
6	7.47	<i>d</i>	15.8	140.91	<i>d</i>
7	—	—	—	128.20	<i>s</i>
8	7.15	<i>s</i>	<i>br</i>	115.01	<i>d</i>
9	—	—	—	146.45	<i>s</i>
10	—	—	—	148.72	<i>s</i>
11	6.87	<i>s</i>	<i>br</i>	116.41	<i>d</i>
12	7.02	<i>d</i>	4.3, <i>br</i>	122.37	<i>d</i>

*This signal is unusually small and broad.

Compared to **1** the new pigment shows very similar ^1H NMR signals of three aromatic and two *trans*-olefinic protons belonging to the steryl residue (Table 1) [7]. The spectrum further contains two singlets, one of an olefinic proton (3-H) at $\delta_{\text{H}} = 5.77$ and another of a methyl group (1- H_3) at $\delta_{\text{H}} = 2.10$. These signals were directly correlated by a ^1H , ^{13}C NMR spectrum to carbon signals at $\delta_{\text{C}} = 101.08$ and $\delta_{\text{C}} = 26.59$, respectively. The ^{13}C NMR spectrum additionally presents the signals of a carbonyl carbon (C-2) at $\delta_{\text{C}} = 197.64$. From its correlations in the long-range ^1H , ^{13}C NMR spectrum the carbonyl carbon (C-2) is flanked by the methyl group (C-1) and the olefinic methine (C-3).

According to the HR-EI mass spectrometry of the molecular ion at m/z 220, the remaining signal in the ^{13}C NMR spectrum, C-4 at $\delta_{\text{C}} = 179.00$, must bear the hydroxyl group which is necessary to complete the elemental composition $\text{C}_{12}\text{H}_{12}\text{O}_4$. This signal is correlated by signals in the long-range ^1H , ^{13}C NMR spectrum to the *trans* double bond methines 5-H and 6-H. Since no alternative connection remains, this completes the structure of **2**, although no correlation signals could be observed indicating the connection between C-3 and C-4. The ^{13}C NMR data for the keto-enol part nearly coincide with those in 1,7-diphenyl-5-hydroxy-4,6-heptadien-3-one ($\delta_{\text{C}} = 199, 101$ and 177 in CDCl_3) [8]. Typically for keto-enol functions the carbonyl band appears at 1635 cm^{-1} in the IR spectrum.

Structure **2** nicely explains the disappearance of the 3-H signal in the ^1H NMR spectrum of hispolon in methanol- d_4 by H/D exchange due to keto-enol tautomerism and it fully agrees with all further spectroscopic data and the chemical properties given in the Experimental. No signals for the 1,3-diketo form were present in the NMR spectra.

EXPERIMENTAL

Fungal material. Fruit bodies of *I. hispidus* were collected in the vicinity of Greifswald (Germany) in August

1993 and 1994. The mushrooms were cut into small pieces, dried, and stored at room temp. in air-tight containers. The fungal material was kindly identified by Prof. Dr H. Kreisel, Department of Biology, EMA-University, Greifswald. A voucher specimen is deposited at the institute of Pharmaceutical Biology, EMA-University, Greifswald.

General methods. Mps were determined by differential scanning calorimetry on a Shimadzu DSC-50. NMR spectra were recorded in $\text{Me}_2\text{CO}-d_6$ or $\text{MeOH}-d_4$. TMS was used as int. standard. EI-MS spectra were measured on a Finnigan MAT 95 spectrometer. CC was performed on Silica gel 60 (0.040–0.060 mm, Merck) and Sephadex LH-20 (Pharmacia). For analyt. and prep. TLC (0.25 mm, 20×20 cm, Merck) and HPTLC (0.2 mm, 10×5 cm, Merck) Silica gel 60 F₂₅₄ was used. For analyt. HPLC a Hewlett Packard HP 1090 system, series II [oven temp. 27° ; column with Techsphere 5-ODS (250×4 mm, 5 nm; HPLC Technology, U.K.); flow rate: 1.0 ml min; detection: UV absorption at 254 nm] was used. For CC, TLC and HPLC the following solvents or mixts were used: A = MeOH; B = EtCO_2H –toluene– HCO_2H (7:5:0.2); C = MeCO_2H –*n*-hexane– HCO_2H (10:5:0.1); D = $\text{MeOH}-\text{CH}_2\text{Cl}_2$ (1:1); E = $\text{CH}_3\text{CN}-\text{H}_2\text{O}$ (4:1).

Extraction. The freeze-dried and pulverized material of *I. hispidus* (100 g) was defatted with *n*-hexane (3×700 ml) in a Soxhlet for 24 hr, dried and then extracted with 80% EtOH (3×1.2 l) under shaking for 15 hr at room temp. The filtered ethanolic extracts were combined, and evapd *in vacuo*. The remaining aq. mixt. was repeatedly partitioned with $\text{H}_2\text{O}-\text{EtOAc}$ (2:1). The EtOAc layers were dried with Na_2SO_4 , filtered, and evapd under red. pres. to give 13 g brown residue.

Isolation of hispidin (1). In a typical run, 100 mg dried EtOAc extract was dissolved in MeOH and sep'd on a column of Sephadex LH-20 (1×74 cm) with solvent A at 30 ml hr^{-1} . Frs of 5 ml were collected and analysed by analyt. TLC using solvent B and visualization with modified Ehrlich reagent (0.8 g *p*-dimethylaminobenzaldehyde, 30 ml EtOH, 10 ml HCl). Frs 22–24 contained **1** as major compound (R_f 0.16 with solvent B; R_f 0.19 with solvent C). They were pooled, and the solvent was evapd. Compound **1** was crystallized from MeOH to afford 6 mg pure compound as fine yellow needles.

Isolation of hispolon (2). About 1 g dried EtOAc extract was dissolved in EtOH, mixed with kieselguhr (*ca* 500 mg), dried, and applied to a column of silica gel (3×20 cm, 40 g), which was eluted with solvent B at 32 ml hr^{-1} . Frs of 8 ml were collected and monitored by analyt. TLC using solvent C. The substances were detected by UV absorption at 254 and 360 nm and further discriminated by staining with modified Ehrlich reagent, anisaldehyde/ H_2SO_4 reagent (0.5 ml anisaldehyde, 85 ml MeOH, 5 ml H_2SO_4 , 10 ml HOAc) or dinitrophenylhydrazine reagent (0.1 g 2,4-dinitrophenylhydrazine, 99 ml MeOH, 1 ml HCl). Frs 12–18 contained **2** as major component (R_f 0.56 with solvent B; R_f 0.45 with solvent C). They were combined, and the solvent was evapd *in vacuo*, yielding 800 mg (from 13 g dried EtOAc extract by

repeated procedure) of a yellow solid which was further purified by repeated gel chromatography on Sephadex LH 20 (1 × 40 cm, 15 g), with solvent D at 10 ml hr⁻¹. Frs of 4 ml were analysed by TLC. Those containing **2** were pooled, evapd *in vacuo*, and subjected (90 mg/plate, 3 ×) to prep. TLC on Silica gel 60 F₂₅₄ using solvent C. The zone of **2** was eluted with MeOH-EtOAc (1:1). The solvent was evapd under red. pres. to yield 60 mg pigment.

Both fresh and freshly lyophilized fungi were extracted with 80% EtOH. The ethanolic extracts were immediately concd under red. pres. After enrichment by HPTLC on Silica gel 60 F₂₅₄ with solvent B the bands of **2** were eluted with MeOH-EtOAc (1:1) and compared with the pure pigment by HPLC with solvent E.

Hispidin [6-(3',4'-dihydroxystyryl)-4-hydroxy-2-pyrone] (**1**). Mp 258°, crystallized from MeOH; UV, IR, ¹H NMR and MS data were in agreement with those reported for **1** [2, 7]. ¹³C NMR (100 MHz, MeOH-d₄): δ_c (ppm) 173.50 (s, C-2), 167.73 (s, C-4), 162.05 (s, C-6), 148.66 (s, C-11), 146.78 (s, C-12), 137.30 (d, C-8), 128.86 (s, C-9), 121.93 (d, C-14), 116.91 (d, C-7), 116.57 (d, C-13), 114.86 (d, C-10), 101.81 (d, C-5), ca 90 (m, small signal, C-3).

Hispolon [6-(9,10-dihydroxyphenyl)-3,5(E)-hexadien-4-ol-2-one] (**2**). Mp 137°; UV λ_{max}^{MeOH} nm (ε): 373 (4.35), 258 (3.69); IR ν_{max}^{KBr} cm⁻¹: 3363 (br), 3199 (br), 1717 (m), 1635, 1598, 584, 1521, 1443 (all s), 1362 (m), 1303 (s), 1282 (s), 1253 (s), 1111 (s), 967, 941, 808 (m). ¹H and ¹³C NMR

(Table 1); EI-MS (145°) m/z (%) 220 [M]⁺ (57), 202 [M - H₂O]⁺ (100), 187 [M - H₂O - Me]⁺ (67), 177 [M - CH₃ - C=O]⁺ (75), 163 [M - C₂H₅ - C=O]⁺ (94), 159 (57), 145 (42), 131 (50), 71 (44), 57 (74), 55 (44). (+)-DCI (NH₃) m/z (%) = 238 [M + NH₄]⁺ (100), 221 [M + H]⁺ (58); C₁₂H₁₂O₄ calc. 220.0736, found 220.0731 (EI-MS).

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