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A CYCLOPENTABENZOPYRANONE PRODUCED BY THE FUNGUS *HETEROBASIDION ANNOSUM* IN DUAL CULTURES

JOHANN SONNENBICHLER, VIKTOR LAMM, ALFRED GIEREN, OTTMAR HOLDENRIEDER* and HERMAN LOTTER†

Max Planck Institut fuer Biochemie, D-8033 Martinsried, West Germany; *Lehrstuhl fuer Forstbotanik, Universitaet Muenchen, West Germany; †Institut fuer pharmazeutische Biologie, Universitaet Muenchen, West Germany

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Key Word Index—*Heterobasidion annosum*; Basidiomycetes; antagonism; cyclopentabenzopyranone; spectral analyses; X-ray strucaure analysis.

Abstract—A crystalline compound secreted by hyphae of *Heterobasidion annosum* in the presence of the antagonistic fungi *Radulomyces confluens*, *Gloeophyllum abietinum*, *Trametes versicolor* or *Nectria fuckeliana* was isolated. By both spectral and X-ray structure analysis, the compound was identified as 7,8-dihydro-9-hydroxy-5,7,7-trimethylcyclopenta-(g)-2-benzopyran-1(6*H*)-one.

INTRODUCTION

Heterobasidion annosum (Syn. *Fomes annosus*) is one of the most destructive basidiomycete pathogens in the coniferous forests of the world. The infection process is primarily initiated by basidiospores which infect the stump surface of fresh cut conifers. From there the mycelium spreads into the wood, grows along the roots and infects living trees by root contacts. A prophylactic treatment of the stump surfaces with propagules of the basidiomycete *Phlebiopsis* (*Peniophora*) *gigantea* can be successfully used in pine plantations to prevent *Heterobasidion* expansion [1]. During *in vitro* studies on such antagonistic effects, it was observed [2, 3] that, on the hyphae of three *H. annosum* strains, crystals were formed if they were grown in dual cultures with at least 12 different fungal species. This crystal formation was in general associated with termination of growth by both species just after contact or with the appearance of an inhibitory zone. After isolation of the crystals from the aerial mycelium of *H. annosum*, which was grown together with *Radulomyces confluens*, *Gloeophyllum abietinum*, *Trametes versicolor* or *Nectria fuckeliana*, the structure of the crystalline compound was elucidated.

RESULTS AND DISCUSSION

Crystals produced by *Heterobasidion annosum* in the presence of *Radulomyces confluens*, *Gloeophyllum abietinum*, *Trametes versicolor* or *Nectria fuckeliana*, have been isolated from cultures grown in Petri dishes. The colorless needles are soluble in solvents, such as chloroform, acetone and ethanol, and insoluble in water and dilute acids and alkali. Purification can be done by Si gel chromatography, the R_f being 0.68 in chloroform–acetone (14:3). After recrystallization in methanol a mp of 121.5° was determined.

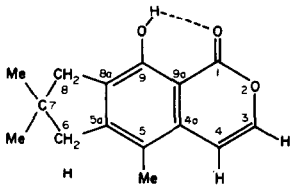
By high resolution mass spectroscopy the molecular peak of the compound was found to be M^+ 244.1095, corresponding to $C_{15}H_{16}O_3$ (calc. 244.1099). This formula again is additionally confirmed by the high resolution mass peak *m/z* 216.1133 ($C_{14}H_{16}O_2$) which originates by the splitting of a carbonyl from the molecular ion. By analysis of all spectroscopic data we assigned formula **1** as an isocoumarin derivative.

Spectral analyses

The UV absorptions of **1** are listed in Table 1 and, compared to similar data for isocoumarin and 8-hydroxy-3-methylisocoumarin (**2a**) isolated from *Marasmius ramealis* [4], a good correspondence can be seen. In the IR spectrum of **1**, a broad band can be seen at 3420 cm^{-1} indicating hydrogen bonding of a hydroxyl group. The sharp, strong IR absorption at 1675 cm^{-1} is characteristic for an α,β-unsaturated lactone. Medium intense absorptions at 1445 and 1335 cm^{-1} and 1240 and 1228 cm^{-1} can be correlated to stretching vibrations for –C–O and C=C–O groups, respectively. The IR spectrum of 8-hydroxy-3-methyl-2-benzopyran-1-one (**2a**) [4] by comparison looks quite similar, as concerns the most intense absorptions: 3480 (m), 1685 (st), 1465 (m), 1320 (m), 1230

Table 1. UV spectral data of isocoumarins

Compound		Ultraviolet absorptions				
1	λ_{max}	231	(239)	260	273	347
	ε	4.41	(4.20)	4.02	3.74	3.60
8-Hydroxy-3-methyl-2-	λ_{max}	228	234.5	256	(271)	341
benzopyran-1-one (**2a**) [4]	ε	4.26	4.28	4.01	(3.77)	3.77
Isocoumarin	λ_{max}	228	239	253	261	318
	ε	4.47	4.22	3.86	3.87	3.58



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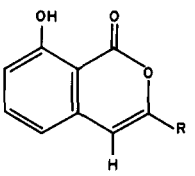

2a R = Me
2b R = H

(m) and 1220 (m) cm^{-1}. In **1** very sharp absorptions at 2920 and 1140 cm^{-1} furthermore signalize $-CH_2-$ groups and an isopropyl moiety.

The ^1H NMR and ^{13}C NMR data are listed in Tables 2 and 3 and compared to these of **2b** [5]. The signal of a phenolic hydroxyl arises at δ 11.06. This resonance disappears by addition of D_2O and is lowfield shifted by a hydrogen bonding to the neighbouring carbonyl group. Two doublets at δ 6.62 and 7.18 belong to the hydrogens at C-4 and C-3, respectively, in **1**. The corresponding resonances in **2b** appear at δ 6.51 and 7.23 with identical coupling constants. The presence of the aromatic methyl group is indicated by a singlet at δ 2.23. The observation of the two signals at δ 2.80 and 2.78 is characteristic for the presence of methylene groups adjacent to double bonds. These, together with an isopropyl moiety at δ 1.19, complete the cyclopentabenzopyranone system.

In the ^{13}C NMR spectrum the dimethylcyclopentane moiety is evident by the resonances at δ 29.1, 39.5, 43.8 and 48.0. The signal at δ 166.9 is characteristic for esters and lactones. Most important are the aromatic carbon resonances. The theoretical shift calculation according to Levy and Nelson [6] also works excellently for substituted benzopyranones, as proved for a series of a coumarins and 1-benzopyrano-4-ones. These increment calculations are also very sensitive to methyl substitutions and, in general for aromatic carbon atoms, serious deviations cannot be tolerated. These observed and calculated δ-values for **1** are listed in Table 3 and give good coincidence to the structure proposed. Only the value for C-5 shows a deviation of δ 3.8. It could, however, not be decided by NMR whether the cyclopentane moiety is in a linear or angular annelation to the aromatic ring. This question was answered by X-ray structure analysis.

Table 2. ^1H NMR spectrum of **1** and for comparison the corresponding signals of **2b** [5] in $CDCl_3$, TMS as int. standard (rel. int. in parentheses), spin states and coupling constants (Hz)

1	2b
11.06 (1), *s*, (exchangeable by D_2O)	10.99 (1), *s*,
7.18 (1), *d*, $J = 6 + 0.3$ (tr/long range)	7.23 (1), *d*, $J = 6$
6.62 (1), *d*, $J = 6 + 0.8$ (tr/long range)	6.51 (1), *d*, $J = 6$
2.80 (2), *d*	
2.78 (2), *dd*, $J = 0.8 + 0.3$	
2.23 (3), *s*	
1.19 (6), *s*	

Table 3. Observed and calculated ^{13}C NMR spectrum of **1** in $CDCl_3$, TMS as int. standard, spin states analysed by off-resonance

	Observed	Calculated
C-1	166.9 *s*	158–175
C-3	142.5 *d*	141.6
C-4	105.4 *d*	106.3
C-4A	133.3 *s*	134.1
C-5	119.1 *s*	122.9
C-5A	154.1 *s*	151.8
C-6	43.8 *t*	40–50
C-7	39.5 *s*	30–40
C-8	48.0 *t*	40–50
C-8A	129.3 *s*	128.2
C-9	155.9 *s*	154.5
C-9A	105.9 *s*	108.4
Aliphatic methyls	29.1 *q*	27
Aromatic methyl	14.2 *q*	15

X-ray structure analysis

The title compound crystallizes in the monoclinic space group *I2/a* (No. 15); $a = 20.922$ (4), $b = 13.930$ (4), $c = 8.636$ (2) Å, $\gamma = 87.06$ (2)°, $V = 2522.0$ Å^3, $D_0 = 1.29$, $D_x = 1.287$ Mg/m^3, $Z = 8$.

Figure 1 shows the molecular structure and bond distances. With the exception of the dimethylmethylene group of the cyclopentene ring and some hydrogens, the molecule is planar. The cyclopentene ring is in an envelope conformation with the dimethyl substituted carbon on the tip of the flap. A chelated hydrogen bond is formed between the hydroxyl group as a donor and the carbonyl oxygen as an acceptor. The bond distances show an aromatic phenol system and a π-interaction in the α,β-unsaturated enol ester system of the six-membered lactone ring. The C–C bond lengths between these two π systems (1.45 Å on average) show a slight shortening indicating some π-interactions.

The mass spectrum of **2b** for comparison is characterized by three consecutive losses of 28 mass units [5]. This is also true for the fragmentation pattern of **1**: *m/z* 244 → 216; *m/z* 201 → 173 → 145. Characteristic for the methyl substitution is the repeated loss of 15 mass units: *m/z* 244 → 229; *m/z* 216 → 201. Furthermore, the series *m/z* 91 → 65 and *m/z* 77 → 51 confirm the presence of an aromatic methyl substituted ring.

With dilute sodium hydroxide, **1**, forms a yellow

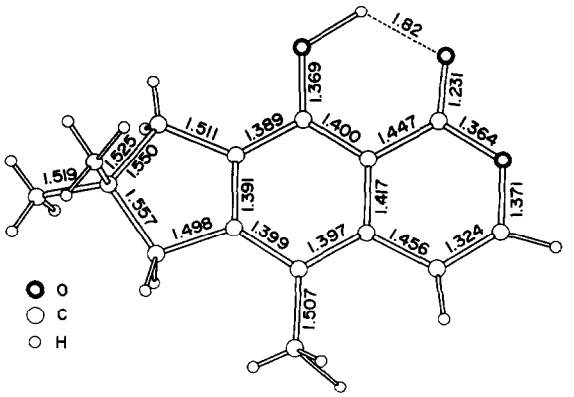

Fig. 1. Molecular structure of 7,8-dihydro-9-hydroxy-5,7,7-trimethylcyclopenta-(g)-2-benzopyran-1 (6*H*)-one. E.s.d.s of given bond lengths are: for C–C and C–O, 0.006–0.010 Å; for H–O, 0.10 Å.

precipitate which is soluble in methanol but very sensitive to autoxidation. The NMR spectra in pyridine indicate the cleavage of the lactone ring by alkali treatment producing a carboxylate and an enolate structure: no NMR signals for an aldehyde structure could be observed.

Compound **1a** has not yet been described in detail in the literature. However, we understand that D. M. X. Donnelly, J. O'Reilley, J. Polonsky and G. W. van Eijk reported an identical isocoumarin from *Heterobasidion annosum* during the *IUPAC 12th Int. Symp. Chem. Nat. Prod.*, Tenerife, 1980. The identity of data could not be checked because they have not been published up to now.

EXPERIMENTAL

Heterobasidion annosum (Fr.) Bref. (*Fomes annosus* P. Karst.) strain L1, *Radulomyces confluens* (Fr.) M. P. Christ., *Cloephyllum abietinum* (Bull. ex. Fr.) P. Karst., *Trametes versicolor* (L. ex. Fr.) Pil. and *Nectria fuckeliana* Booth, were isolated from fruiting bodies on *Picea abies*. The fungi were grown in Petri dishes on 2% malt extract–agar (Merck, Darmstadt) for 4–6 weeks at 21° in the dark. The crystals containing aerial mycelium of *H. annosum* were scraped off with H_2O. The suspension was filtered, the residue washed with H_2O and dried with Si gel. After solubilization in $CHCl_3$ the extract was evaporated and purification was performed by TLC on Kieselgel 60 (Merck) in $CHCl_3$–Me_2CO (14:3) R_f 0.68; R_f in C_6H_6 0.22.

^1H NMR and ^{13}C NMR spectra were measured in $CDCl_3$ with TMS as a standard. UV spectra were recorded in EtOH.

For the X-ray structure analysis the data collection was performed with the aid of a Nicolet R3m diffractometer. A crystal with dimensions $0.2 \times 0.1 \times 0.6$ mm, isolated from C_6H_6, was used. The cell dimensions were obtained by least squares from the setting angles of 14 reflections. With Cu K_α radiation (graphite monochromator) 3117 reflections were measured in the $\omega/2\theta$ scan mode. As usual, corrections were applied for L_p but not for the absorption [$\mu(\text{Cu}\,K_\alpha) = 0.64\,\text{mm}^{-1}$]. After eliminating the unobserved reflections [$I < 2\sigma(I)$] 1194 independent structure factors were obtained by averaging the symmetry equivalent ones.

All computations for structure solution and refinement were carried out on a Vax 11/782 computer using the program SHELX 76 [7]. The structure was solved by direct methods. For phase determination the 232 highest E-values were used. In an E-Fourier map resulting from the best phase set, according to the criterion of consistency, a partial structure containing 14 of 18 nonhydrogen atoms could be recognized. By a successive Fourier synthesis all nonhydrogen atoms were localized. They could also be found in an E-Fourier map calculated with the third best phase set of an alternative phase determination, in which the five highest E-values were omitted. After some steps of least squares refinement—first with isotropic and later with anisotropic temp. parameters—all hydrogen atoms were visible in difference Fourier maps and were included with isotropic temp. parameters in the refinement. With unit weights it converged to $R = 0.058$.

The lists of structure factors, positional and temp. parameters of all atoms, bond distances, bond angles and torsion angles have been deposited with the Cambridge Crystallographic Data Centre.

High resolution MS were recorded on a Varian MAT 311 A instrument. Mass spectroscopic fragmentation of the isolated **1a** at 240° [only the peaks with more than 15% rel. int. (in parentheses) are listed]: 244 (90), 229 (50), 216 (100), 201 (30), 173 (20), 145 (16), 129 (28), 128 (41), 115 (40), 91 (30), 77 (33), 65 (20), 63 (20), 51 (28), 41 (31), 39 (45).

The IR spectra were recorded in KBr. Absorption bands (cm^{-1}): 3420 (br m), 2920 (st), 1675 (st), 1628 (w), 1445 (m), 1335 (m), 1240 (w), 1228 (w), 1140 (m), 804 (m), 742 (m).

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