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Cladobotryal: a Fungal Metabolite with a Novel Ring System

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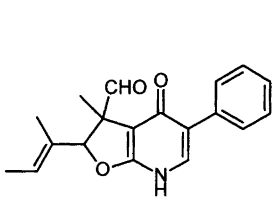
In screening for antifungal metabolites a novel compound, cladobotryal, was isolated from the mycoparasitic fungus *Cladobotryum varium*. Its structure was established as (+)-(2*R**,3*R**)-2-[(*Z*)-2-buten-2-yl]-3,7-dihydro-3-formyl-3-methyl-5-phenylfuro[2,3-*b*]pyridin-4(2*H*)-one on the basis of spectroscopic evidence and single crystal X-ray analysis of its methyl hemiacetal. The fused furo[2,3-*b*]pyridinone skeleton of cladobotryal seems unprecedented within the chemical literature.

During the course of our search for fungal metabolites with growth inhibitory activity we observed a marked and curiously selective effect of the mycoparasitic fungus *Cladobotryum varium* Nees: Fries (CBS 331.95) (isolated from *Trametes versicolor*) on phytopathogenic species of the class Oomyceta. Propagation of the *Cladobotryum* fungus on solid agar medium, followed by solvent extraction and chromatographic purification (see Experimental) afforded a homogeneous, amorphous metabolite, *cladobotryal*, possessing a distinct inhibitory effect on the growth of various plant pathogens belonging to Oomyceta. We report on the isolation and structure elucidation of cladobotryal and comment on the novelty of its molecular framework and on previously reported *Cladobotryum*-metabolites with antifungal activity.

Structure elucidation

A homogeneous specimen of cladobotryal, procured as described in the Experimental, exhibited a high-resolution EI-MS in accord with the molecular composition $C_{19}H_{19}NO_3$, with an index of hydrogen deficiency of eleven. In its ^{13}C NMR spectrum signals corresponding to seventeen magnetically non-equivalent carbon atoms were observable (Table 1). According to δ-values, multiplicity analysis (DEPT), and IR data, the nineteen carbon atoms could be assigned to three C-methyl, one methine (oxygenated), one quaternary, one formyl (ν_{max} 1728 cm^{-1}), one carbonyl (ν_{max} 1648 cm^{-1}) and twelve unsaturated carbon atoms. Consequently, cladobotryal must contain three rings. The ^1H NMR spectrum (Table 1) served to confirm and substantiate the above assignments. 2D NMR measurements (HSQC and HMBC) provided sufficient additional evidence to render **1**, and the regioisomer **2**, the only, though equally acceptable general structures.



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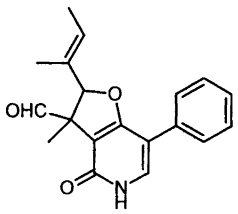

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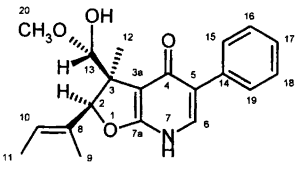

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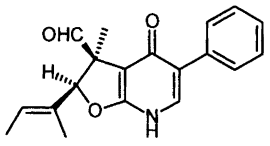

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Table 1. ^{1}H and ^{13}C NMR data for cladobotryal (**4**).

C[a]	^{13}C	^{1}H
2	93.8 (d)	4.97 (s)
3	57.9 (s)	
3a	105.6 (s)	
4	159.5 (s)	
5	121.9 (s)	
6	148.4 (d)	7.87 (s)
7a	167.6 (s)	
8	130.4 (s)	
9	12.2 (q)	1.55 (br, s)
10	126.1 (d)	5.84 (q, J=6.8 Hz)
11	13.1 (q)	1.70 (d, J=6.8 Hz)
12	21.7 (q)	1.64 (s)
13	202.7 (d)	9.71 (s)
14	133.9 (s)	
15/19	129.3 (d)	7.48 (m)
16/18	128.9 (d)	7.48 (m)
17	127.8 (d)	7.38 (m)

[a]The numbering as indicated in formula **3**.

A choice between the regioisomers was rendered feasible when it was noticed that a hot methanolic solution of cladobotryal on being cooled deposited well shaped, colourless needles with the elemental composition $C_{20}H_{23}NO_4$ and obviously consisting of a methyl hemiacetal of cladobotryal (**3**). A single-crystal X-ray analysis of **3** established its structure (Fig. 1) and hence that of cladobotryal as (+)-(2*R**,3*R**)-2-[(*Z*)-2-buten-2-yl]-3,7-dihydro-3-formyl-3-methyl-5-phenylfuro[2,3-*b*]pyridin-4(2*H*)-one, represented by **4** (or its enantiomer), unequivocally excluding the type **2** regioisomer. Owing to the rather poor reflecting power of the crystal, the data were insufficient for a detailed comparison of the bond lengths and angles with those reported for related structures.

Biological activity. In vitro growth inhibitory activity was assessed by means of agar diffusion assays as described previously.[1] Cladobotryal exhibited a marked effect against the phytopathogenic fungi *Phytophthora cryptogea* and *Pythium ultimum* (Oomyceta), whereas the growth of ascomycetes, various imperfect fungi, yeast species, and bacteria was virtually unaffected.

Discussion

Mycoparasitic *Cladobotryum*-species have been repeatedly reported as sources of metabolites possessing antimicrobial activity. They comprise a polypeptide of unknown structure,[2] two antifungal α-pyrone aldehydes (cladobotrin I and II),[3] a trisubstituted furan aldehyde (brunnescin), and a substituted pyridinedione (flavipucin).[4] The tetracyclic hypomycetin was recently reported from our laboratories as a metabolite from the mycophilic fungus *Hypomyces aurantius*,[5] the anamorphous state of which has been recognized as *C. varium* Nees: Friis, the very source of cladobotryal.

Obviously *Cladobotryum* species are characterized by a remarkably diverse synthetic proficiency, now shown to include the assembly of the hitherto unknown, fused furo[2,3-*b*]pyridin-4-one system typical of cladobotryal. Its biosynthetic origin remains obscure, though phenylalanine and two molecules of mevalonic acid are likely donors of the nineteen carbon atoms in cladobotryal.

Experimental

General. ^{1}H and ^{13}C NMR data were acquired at 300 K on a Bruker AC300 or DRX400 instrument. Chemical shifts (δ), measured in $CDCl_3$, are in ppm relative to residual solvent signals at δ 7.27 (^{1}H) and δ 77.0 (^{13}C); coupling constants (J) are given in Hz. The EI mass spectrum, at 70 eV ionisation potential, was recorded on a JEOL AX505W instrument, and is presented as *m/z* (% rel. int.). X-Ray data were collected on an Enraf–Nonius CAD4F single crystal diffractometer.

Production. Cladobotryum varium (CBS 331.95) was propagated in Petri dishes (9 cm diameter) each containing 20 ml of potato dextrose agar (39 g l^{-1}) for 7 days at 26 °C. Agar plugs with mycelial growth were used to inoculate Petri dishes containing 20 ml of malt extract agar [MEA; malt extract (20 g l^{-1}); peptone

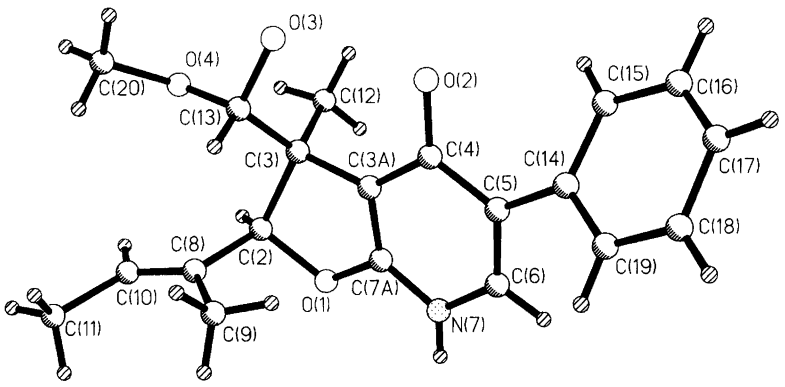

Fig. 1. Crystal structure of cladobotryal methyl hemiacetal (**3**).

(1 g l^{-1}); glucose (20 g l^{-1})]. The fermentation on MEA was conducted for 12–14 days at 26 °C.

Extraction. The contents of 94 Petri dishes were extracted with mechanical stirring with 2.5 l of EtOH for 30 min. The solids were filtered off and extracted with a second 2.5 l portion of EtOH for 2 h. After filtration the combined EtOH-extracts were evaporated *in vacuo*. The residue was suspended in water (500 ml) and extracted twice with 500 ml portions of EtOAc. The combined organic phases were dried (freezing and removal of the separated ice) and the solvent evaporated.

Purification. The EtOAc-extract was dissolved in CH_2Cl_2–MeOH (9:1) and in four portions subjected to silica gel column chromatography [Si 60 40–63 μm, Lobar size B (Merck), eluting at a flow rate of 7.5 ml/min with a heptane–*tert*-butyl methyl ether gradient 90:10 → 0:100 (30 min) → 0:100 (10 min)]. The effluent was monitored by UV absorption at 240 nm. The peak eluting at ≈35 min was collected to yield cladobotryal as an amorphous, colourless powder (178 mg). ^1H and ^{13}C NMR data are presented in Table 1; HR-EIMS: found 309.1367, calc. for $C_{19}H_{19}NO_3$ 309.1364; EI-MS: 309 (31%), 292 (13), 280 (100), 264 (10), 252 (20), 238 (10), 226 (14), 225 (9), 200 (5) and 172 (5). IR (KBr): 3433, 2929, 1728, 1648, 1596, 1560, 1500–1375 (multiple bands), 1294, and 1066 cm^{-1}. $[\alpha]_D^{24} = +65°$ ($c = 0.8$, $CHCl_3$). UV [λ_{max} (log ε) in MeOH]: 234 nm (4.35), ca. 260 (4.04).

Crystallisation from MeOH afforded the methanol hemiacetal **3** as colourless needles, m.p. 198–199 °C; C, H, N: $C_{20}H_{23}NO_4 \cdot 0.5\ CH_3OH$; $[\alpha]_D^{22} = +46°$ ($c = 0.7$, MeOH); NMR: upon dissolution in $CDCl_3$ **3** exhibited a complex ^1H NMR spectrum due to the presence of diastereomeric hemiacetals as well as the free aldehyde. The sample obtained by evaporation and redissolution in $CDCl_3$ exhibited NMR spectra identical with those of cladobotryal.

X-Ray techniques. Crystal and experimental data for cladobotryal methyl hemiacetal (**3**) are listed in Tables 2 and 3. The crystals were cooled to 120 K using the Cryostream nitrogen gas cooler system.[6] The unit cell was derived from a least-squares fit of refined diffractometer setting angles for 25 reflections. Four standards were measured for intensity and orientation control after every 4 h. The intensities were corrected for Lorentz and polarization effects. The structure was solved by direct methods and refined by full-matrix least-squares techniques. Unfortunately, the reflecting power of the crystal was rather poor. The non-hydrogen atoms were refined isotropically. In order to reduce the number of variables, the thermal parameters of C(2), C(3)···C(7a), N(7), O(1) and O(2) were constrained to be identical and so were the temperature factors of C(14)···C(19) and those of C(8), C(10), C(13), O(3) and O(4). The temperature factors of the methyl carbons were identical. These constraints were chosen after refinement with all non-hydrogen atoms having individual thermal parameters. The hydrogen atoms were all at generated positions using a riding model with C–H = 0.95–0.98 Å and N–H = 0.88 Å and fixed thermal parameters [$U(H) = 1.4 \times U$ for attached atoms]. The origin was fixed by use of least-squares restraints.[7] The correct absolute structure could not be determined reliably. The crystallographic computations were performed with SHELXS86[8] and SHELXL93.[9] The atomic scattering factors were taken from the literature.[10] The SHELXTL program[11] was used for illustration and PLATON[12] for molecular geometry calculations.

Table 2. Crystal and experimental data for cladobotryal methyl hemiacetal (**3**).

Formula	$C_{20}H_{23}NO_4$
Formula weight	341.41
Crystal system	Trigonal
Space group	*R*3
Unit-cell dimensions	
a/Å	19.678(6)
b/Å	19.678(6)
c/Å	13.192(5)
Unit-cell volume, *V*/Å^3	4424(3)
Formula units per unit cell, *Z*	9
F(000)	1629
Calculated density, D_x/g cm^{-3}	1.15
Radiation	Mo Kα
Wavelength, λ/Å	0.71073
Linear absorption coefficient/cm^{-1}	0.8
Temperature, *T*/K	120
Crystal description	Colourless
Crystal size/mm	0.20 × 0.08 × 0.08
Diffractometer	Enraf–Nonius CAD-4F
Unit-cell determination	
No. of reflections used	25
θ-Range/°	5.0–11.1
Intensity data collection	
θ_{max}/°	25
Range of *h*	−27–0
Range of *k*	0–23
Range of *l*	−18–0
Scan mode	ω
Scan range, Δω	0.35 + 0.35 tan θ
Total number of unique reflections	985
No. of independent reflections, [$I > 2\sigma(I)$]	485
Corrections	Lorenz polarization
Structure refinement	
Minimization of isotropic thermal parameters	$\Sigma w(\|F_o\|^2 - \|F_c\|^2)^2$
No. of refined parameters	All atoms
Weighting scheme	$[\sigma^2(F_o^2) + (0.1594P)^2 + 6.46P]^{-1}$ [a]
$R = \Sigma\|\|F_o\| - \|F_c\|\|/\Sigma\|F_o\|$	0.1057 (485 reflections)
$wR2 = [\Sigma\|F_o^2 - F_c^2\|^2/\Sigma wF_o^4]^{1/2}$	0.3206 (985 reflections)
$S = [\Sigma w(\|F_o\|^2 - \|F_c\|^2)^2/(N_{obs} - N_{var})]^{1/2}$	1.09
Final $(\Delta/\sigma)_{max}$	0.07
Final $\Delta\rho_{min}$ and $\Delta\rho_{max}/e$ Å^{-3}	−0.28 and 0.52

[a] $P = (F_o^2 + 2F_c^2)/3$.

Table 3. Fractional atomic coordinates and equivalent isotropic thermal parameters for **3** (in Å^2).

Atom	*x*	*y*	*z*	*U*
O(1)	0.4180(7)	0.4560(7)	0.0937(9)	0.0460(13)
O(2)	0.4344(7)	0.5341(7)	0.4330(9)	0.0460(13)
O(3)	0.2909(7)	0.4937(7)	0.3760(8)	0.0436(17)
O(4)	0.2222(7)	0.4632(7)	0.2258(9)	0.0436(17)
N(7)	0.5206(9)	0.4673(9)	0.1864(11)	0.0460(13)
C(2)	0.3402(11)	0.4453(11)	0.1147(14)	0.0460(13)
C(3)	0.3520(11)	0.4936(12)	0.2181(14)	0.0460(13)
C(3a)	0.4227(11)	0.4918(11)	0.2584(15)	0.0460(13)
C(4)	0.4613(11)	0.5116(11)	0.3551(14)	0.0460(13)
C(5)	0.5308(10)	0.5073(11)	0.3634(14)	0.0460(13)
C(6)	0.5574(11)	0.4867(11)	0.2769(14)	0.0460(13)
C(7a)	0.4565(11)	0.4722(11)	0.1832(14)	0.0460(13)
C(8)	0.2806(11)	0.3571(10)	0.1174(14)	0.0436(17)
C(9)	0.2994(13)	0.3059(14)	0.1811(16)	0.063(3)
C(10)	0.2195(10)	0.3322(11)	0.0599(15)	0.0436(17)
C(11)	0.1567(12)	0.2469(12)	0.0484(18)	0.063(3)
C(12)	0.3756(13)	0.5796(13)	0.1895(16)	0.063(3)
C(13)	0.2794(11)	0.4560(11)	0.2807(13)	0.0436(17)
C(14)	0.5703(13)	0.5195(12)	0.4637(16)	0.058(2)
C(15)	0.5870(13)	0.5861(12)	0.5237(15)	0.058(2)
C(16)	0.6288(12)	0.5964(13)	0.6137(16)	0.058(2)
C(17)	0.6540(12)	0.5499(11)	0.6440(16)	0.058(2)
C(18)	0.6425(12)	0.4872(13)	0.5814(15)	0.058(2)
C(19)	0.6013(12)	0.4732(12)	0.4960(16)	0.058(2)
C(20)	0.1420(12)	0.4098(12)	0.2594(17)	0.063(3)

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