Strobilurin N and Two Metabolites Related to Chorismic Acid from the Fruit Bodies of Mycena crocata (Agaricales)

Malcolm S. Buchanan, Wolfgang Steglich, and Timm Anke

Institut für Organische Chemie der Universität, Butenandtstr. 5–13 (Haus F), D-81377 München, Germany. Fax: +49–89–2180–7756. E-mail: wos@cup.uni-muenchen.de

Lehrbereich Biotechnologie der Universität, Paul-Ehrlich-Straße 23, D-67663 Kaiserslautern, Germany

* Author for correspondence and reprint requests

Z. Naturforsch. 54c, 463–468 (1999); received March 18/April 12, 1999

Mycena crocata, Toadstool, Strobilurins, Chorismic Acid Derivatives, Fungicide

Strobilurin N (1), a new member of the strobilurin family of antibiotics, has been isolated from the toadstool Mycena crocata, together with dehydrochorismic acid lactone (5) and 3-[[l-carboxyvinyl]oxy]benzoic acid (6), which are closely related to chorismic acid (10). Their structures were determined mainly by 1H and 13C NMR spectroscopy. Interestingly, strobilurin N is the first strobilurin without antifungal activity.

Introduction

The strobilurins (Anke and Steglich, 1989; Clough, 1993; Anke, 1997; Anke and Steglich, 1998) are naturally occurring β-methoxyacrylates that are produced by various fungi, however, almost exclusively within the class basidiomycetes. These compounds have created great interest because of their significant antifungal activity that is due to an inhibition of mitochondrial respiration. Mycena species are known to be a rich source of this type of antibiotic (Schramm et al., 1978; Bäuerle, 1981; Backens et al., 1988; Anke, 1995; Daferner et al., 1998). Herein we describe the isolation and structure elucidation of a strobilurin N (1) and two compounds related to chorismic acid from fruit bodies of Mycena crocata (Schrad. ex Fr.) Kummer. Strobilurin B (2) has already been isolated from cultures of the same species (Bäuerle and Anke, 1980). The only other known occurrence of a strobilurin in fruit bodies is that of strobilurin G (= "strobilurin D") in M. tintinabulum (Engler et al., 1998).

Material and Methods

General

IR: Perkin Elmer FT-IR 1000 instrument, in KBr. UV: Perkin Elmer Lambda 16 instrument, in MeOH. CD: Jobin Yvon Instruments S. A. CD-6 Dichrograph, in MeOH. NMR: Bruker AMX 600 (1H, 600 MHz; 13C, 150 MHz). EIMS: Finnigan MAT 9Q instrument measured at 70 eV. TLC: over Merck precoated silica gel 60 F254 (0.25 mm on aluminium foil) and visualised under UV light (254 or 366 nm). CC: Silica gel 60 (40–63 µm) and Sephadex LH-20. HPLC: Waters-Millipore with gradient controller M680, two M590 EF pumps and U 6K injector equipped with a Knauer-Vertex column (250 x 16.0 mm) with a precolumn.

Fungal material

Mycena crocata was collected and identified by Dr. N. Arnold in the Platzerkuppe Natural Forest Reservation, Rhön, Germany in October 1997. The saprophytic fungus was found growing on leaves and twigs on the forest floor. A voucher specimen is kept at the Institute of Organic Chemistry, University of Munich.

Tests for biological activities

The assays for antimicrobial (Anke et al., 1977) and cytotoxic (Zapf et al., 1995) activities were carried out as described previously.

Extraction and isolation

When collecting the fresh material it was immediately immersed in MeOH and then stored in a freezer. The residue (3.15 g) obtained after evaporation of the MeOH extract was partitioned be-
tween EtOAc/H₂O, the organic phase was then evaporated and the residue subjected to silica gel column chromatography (gradient from n-hexane to 1:1 n-hexane/EtOAc v/v). Purification of the latter fractions by preparative HPLC on RP-18 stationary phase (gradient from 9:1 H₂O/CH₃CN + 0.1% CF₃COOH to CH₃CN + 0.1% CF₃COOH over 30 min) yielded 4.1 mg of strobilurin N (1), Rᵣ 29.6 min.

The resulting aqueous residue from the first extraction was acidified with 2M HCl to pH 1–2 and again partitioned with EtOAc. Evaporation of the organic phases yielded 800 mg residue which was then applied to a Sephadex LH-20 column eluted with MeOH. Subsequent purification by preparative HPLC on RP-8 stationary phase (gradient from 9:1 H₂O/CH₃CN + 0.1% CF₃COOH to CH₃CN + 0.1% CF₃COOH over 30 min) yielded 24 mg of 5 and 6 mg of 6.

Strobilurin N (1) was obtained as an amorphous solid. – UV, λmax (log ε): 288 nm (3.43), 299 (3.43). – IR, ν: 3436 (OH); 2925, 2854, 1714, 1694, 1682, 1506, 1436, 1277, 1203, 1123, 1089 cm⁻¹. – CD: no effect. – See Table I for NMR data. – EIMS, m/z (rel. int.): 390.1681 (100%, M⁺), C²₁H₂₆O₇ requires 390.1679, 358 (20), 331 (9), 253 (85), 153 (45), 75 (34).

3-Methylene-2-oxo-2,3-dihydro-benzo[1,4]-dioxin-6-carboxylic acid (Delaydrochorismic acid lactone) (5) was obtained as an amorphous solid. – UV, λmax (log ε): 254 nm (3.98), 286 (3.30). – IR, ν: 3429 (OH), 1707, 1683, 1634, 1609, 1593, 1517, 1424, 1384, 1301, 1209, 1142. – See Table II for NMR data. – EIMS, m/z (rel. int.): 206.0210 (100%, M⁺), C₁₀H₈O₅ requires 206.0215, 178 (38), 161 (35), 154 (19), 138 (28), 121 (19), 119 (13), 69 (76), 45 (77).

3-[1-Carboxyvinyl]oxy]benzoic acid (6) was obtained as an amorphous solid. – UV, λmax (log ε): 288 nm (3.00). – IR, ν: 3423 (OH), 1701, 1587, 1438, 1409, 1297, 1268, 1223 cm⁻¹. – ¹H NMR (CHD₂COCD₃, δH at 2.05): δH 7.79 (1H, ddd, J = 8.0, 1.6, 1.0 Hz); 7.63 (1H, ddd, J = 2.6, 1.6, 0.4 Hz); 7.51 (1H, td, J = 8.0, 0.4 Hz); 7.29 (1H, ddd, J = 8.0, 2.6, 1.0 Hz); 5.89 (1H, d, J = 1.8 Hz); 5.21 (1H, d, J = 1.8 Hz). – ¹³C NMR (CD₃COCD₃, δC at 29.8): δC 167.4 (s), 163.9 (br. s), 157.5 (s), 151.1 (br. s), 133.4 (s), 130.8 (d), 125.4 (d), 123.3 (d), 119.3 (d), 108.1 (t). – EIMS, m/z (rel. int.): 208.0371 (2%, M⁺, C₁₀H₈O₅ requires 208.0372), 179 (15), 138 (46), 121 (45), 69 (100), 45 (61).

Results and Discussion

Strobilurin N

Fresh fruit bodies of Mycena crocata were extracted with methanol giving a red-orange solution. Evaporation of the extract, partition of the residue between ethyl acetate and water followed by silica gel column chromatography and HPLC of the ethyl acetate residue gave strobilurin N (1) in very small yield.

Strobilurin N (1) has the molecular formula C₂₁H₂₆O₇ (m/z 390.1681 [M⁺]) which followed from the EIMS. The infrared spectrum showed the presence of hydroxyl and carbonyl groups, while the UV/Vis spectrum (MeOH) indicated chromophores with absorption maxima at λmax 288 and 299 nm.

The ¹H NMR spectrum (Table I) showed a coupled system of three olefinic protons: δH 6.23 (br. d, J = 10.5 Hz), 6.38 (d, J = 16.0 Hz) and 6.48 (dd, J = 16.0, 10.5 Hz). This coupling pattern was also revealed by the H,H-COSY spectrum which further showed an allylic coupling between the olefinic methyl at δH 1.96 and the olefinic proton at δH 6.23. Additionally the ¹H NMR spectrum contained signals for a deshielded olefinic proton [δH 7.43 (s)], three aromatic protons [δH 6.87 (s, 2H); 6.93 (s)], two methoxyls (δH 3.74, 3.85), two tertiary methyls (δH 1.37, 3.85), two tertiary methyls (δH 1.37, 1.41), attached to an oxygen bearing carbon, and an isolated methylene at δH 4.00 (d, J = 11.0 Hz). 4.27 (d, J = 11.0 Hz) attached to an oxygen function. The ¹³C NMR spectrum (Table I) reinforced these assignments and further identified an ester carbonyl (δC 167.9),
five sp²-hybridised quaternary carbons and two 
oxygen bearing tertiary carbons (δC 73.7, 96.4), the 
latter of which is associated with a hemiacetal 
function. All together, resonances were observed 
for twenty-one carbon atoms.

The signals can be assigned to a strobilurin 
structure with an unsaturated side chain, a termi­
nal (E)-β-methoxyacrylate unit and a 1,3,4-trisub­
stituted benzene ring. Furthermore, from the 
chemical shifts of the aromatic carbons, C-2 and 
C-3 (δC 141.5, 141.7), the presence of an isolated 
methylene, and the quaternary carbon at δC 96.4, 
it is clear that this strobilurin contains a dioxane 
ring fused to the aromatic nucleus, similar to strobi­
lurins E (3) (Weber et al., 1990; Bertram et al., 
1996) and M (4) (Daferner et al., 1998). Correla­
tions in an HMBC experiment between one of the 
methylene protons (δH 4.27) and both C-2' (δC 
96.4) and C-3 (δC 141.7) confirmed this deduction. 
From here the structure for strobilurin N (1) was 
earily completed. The remaining NMR signals can 
be assigned to an isopropyl group containing a 
quaternary hydroxyl bearing carbon. This isopro­
pyl group is attached to the dioxane ring at the 
hemiacetalic carbon (δC 96.4). The HMBC corre­
lation between one of the isopropyl methyls (δH 
1.37) and the hemiacetalic carbon made this clear 
and the molecular formula, C₂₁H₂₆O₇, deduced by 
HRMS, with a strong molecular ion m/z 390 [M]⁺ 
(100%) indicated that hydroxyl groups are at­
tached to both of the quaternary oxygenated 
carbons (δC 73.7, 96.4). The relative orientation of 
attachment of the dioxane ring to the benzene nu­
cleus was assumed to be the same as that of strobi­
lurins E (3) and M (4). These were established 
from analysis of the fully coupled ¹³C NMR 
spectrum and long range selective decoupling ex­
periments. Strobilurin E has also been obtained by 
total synthesis (Bertram et al., 1996). As expected, 
measurement of the CD curve of 2 showed no ef­
fect because of epimerisation at the hemiacetalic 
carbon. Strobilurin N (1) could be considered to 
yield strobilurin E (3) by acetal formation with 3­
methyl-2-butenal (compare Bertram et al., 1996).

In the plate diffusion assay no antibacterial (Ba­
cillus brevis, B. subtilis, Enterobacter dissolvens,

<table>
<thead>
<tr>
<th>Site</th>
<th>¹³C</th>
<th>δH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>114.8 d</td>
<td>6.93 (s)</td>
</tr>
<tr>
<td>2</td>
<td>141.5 s</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>141.7 s</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>117.1 d</td>
<td>6.87 (s)</td>
</tr>
<tr>
<td>5</td>
<td>120.6 d</td>
<td>6.87 (s)</td>
</tr>
<tr>
<td>6</td>
<td>132.7 s</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>130.4 d</td>
<td>6.38 (d, 16.0)</td>
</tr>
<tr>
<td>8</td>
<td>125.5 d</td>
<td>6.48 (dd, 16.0, 10.5)</td>
</tr>
<tr>
<td>9</td>
<td>129.7 d</td>
<td>6.23 (br. d, 10.5)</td>
</tr>
<tr>
<td>10</td>
<td>130.8 s</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>110.9 s</td>
<td></td>
</tr>
</tbody>
</table>

a Numbers in parentheses are coupling constants (J) in Hz.
b Assignments confirmed by two-dimensional experiments (COSY, HMQC, HMBC and NOESY).
c CDCl₃ signal at δC 77.0 as reference.
d CHCl₃ signal at δH 7.26 as reference.
Micrococcus luteus) or antifungal (Nematospora coryli, Penicillium notatum, Paecilomyces variotii, Mucor miehei) activities could be observed at concentrations of 50 mg/ml. In addition, strobilurin N did not exhibit cytotoxic activities at the same concentrations towards L1210 (ATCC CCL 219, mouse) and Colo 320 (DSMZ ACC 144, human) cells.

Strobilurin N (1) is the first strobilurin with an (E)-ß-methoxy acrylate acrylate unit that shows no antifungal activity.

Compounds related to chorismic acid

A second EtOAc residue was obtained from the acidified aqueous extract which was left after removal of the neutral components, and after gel permeation through Sephadex LH-20 and subsequent HPLC the colourless acids 5 and 6 were isolated.

The molecular ion of carboxylic acid 5 appears at m/z 206, and high resolution mass spectrometry showed that this corresponds to the molecular formula C_{10}H_{10}O_{4}. The ¹H NMR spectrum (Table II) showed three protons which correspond to a 1,2,4-trisubstituted aromatic ring [δ_H 6.97 (d, J = 8.4 Hz), 7.45 (d, J = 1.3 Hz); 7.62 (dd, J = 8.4, 1.3 Hz)] and two protons of an exo-methylene group [δ_H 4.78, 5.52 (both br. s)]. The ¹³C NMR spectrum (Table II) corroborated these assignments and, furthermore, identified two carbonyls [δ_C 163.8 (br. s) 166.7 (s)] and three quaternary sp²-hybridised carbons; all together resonances were observed from ten carbon atoms. The IR spectrum (KBr) is in agreement with the presence of a hydroxyl (v 3429 cm⁻¹), an unsaturated lactone carboxyl (v 1707 cm⁻¹) and an aromatic acid (v 1683 cm⁻¹). It follows from the above data that carboxylic acid 5 is the lactone of dehydrochorismic acid [Kobayashi et al., 1982].

Carboxylic acid 5 may be derived from chorismic acid (10) by dehydrogenation followed by aromatisation (Asano et al., 1985) and lactonisation of the resulting dehydrochorismic acid (7) (Scheme 1).

The second aromatic carboxylic acid from M. crocata was identified as 3-[(1-carboxy-vinyl)oxy]-benzoic acid (6), a known dehydration product of chorismic acid (10). It can be obtained from 10 by treatment with acetic anhydride in pyridine (Ife et al., 1976; Mattia and Ganem, 1994) or on standing in DMSO solution (Grimshaw et al., 1984) and has been synthesised by Lingens and Sprößler (1967). The spectral data of the natural product were in close agreement with those given in the literature.
Scheme 1: Proposed formation of carboxylic acid 5 from chorismic acid (10)

**Acknowledgements**

We are grateful to Boehringer/Mannheim–Hoffmann La Roche AG for financial support and a Research Fellowship to M. S. B. Dr. N. Arnold kindly collected and identified the fungal material. Thanks are also due to Dr. W. Spahl for measuring mass spectra, Mr. H. Huber for IR and UV measurements, and Mr. A. Werle for technical assistance.


