

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

Malcolm S. Buchanan^a, Wolfgang Steglich^{a,*} and Timm Anke^b

^a 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

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

* Author for correspondence and reprint requests

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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-[1-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* (Schr. 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. tintinnabulum* (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 95Q 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 μ m) 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 \times 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-

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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**), *R*_t = 29.6 min.

The resulting aqueous residue from the first extraction was acidified with 2 M 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 (v/v) + 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, λ_{\max} (log ϵ): 288 nm (3.43), 299 (3.43). – IR, $\tilde{\nu}$: 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 (*Dehydrochorismic acid lactone*) (**5**) was obtained as an amorphous solid. – UV, λ_{\max} (log ϵ): 254 nm (3.98), 286 (3.30). – IR, $\tilde{\nu}$: 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⁺, C₁₀H₆O₅ 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, λ_{\max} (log ϵ): 288 nm (3.00). – IR, $\tilde{\nu}$: 3423 (OH), 1701, 1587, 1438, 1409, 1297, 1268, 1223 cm⁻¹. – ¹H NMR (CHD₂COCD₃, δ_{H} at 2.05): δ_{H} 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, δ_{C} at 29.8): δ_{C} 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).

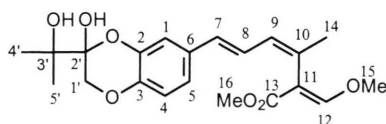
Results and Discussion

Strobilurin N

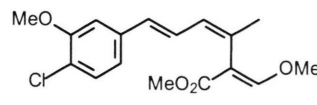
Fresh fruit bodies of *Mycena crocata* were extracted with methanol giving a red-orange solution. Evaporation of the extract, partition of the residue between ethyl acetate and water followed by silica gel column chromatography and HPLC of the ethyl acetate residue gave strobilurin N (**1**) in very 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 λ_{\max} 288 and 299 nm.

The ¹H NMR spectrum (Table I) showed a coupled system of three olefinic protons: δ_{H} 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 δ_{H} 1.96 and the olefinic proton at δ_{H} 6.23. Additionally the ¹H NMR spectrum contained signals for a deshielded olefinic proton [δ_{H} 7.43 (s)], three aromatic protons [δ_{H} 6.87 (s, 2H); 6.93 (s)], two methoxys (δ_{H} 3.74, 3.85), two tertiary methyls (δ_{H} 1.37, 1.41), attached to an oxygen bearing carbon, and an isolated methylene [δ_{H} 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 (δ_{C} 167.9),



1



2

five sp^2 -hybridised quaternary carbons and two oxygen bearing tertiary carbons (δ_C 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 terminal (*E*)- β -methoxyacrylate unit and a 1,3,4-trisubstituted benzene ring. Furthermore, from the chemical shifts of the aromatic carbons, C-2 and C-3 (δ_C 141.5, 141.7), the presence of an isolated methylene, and the quaternary carbon at δ_C 96.4, it is clear that this strobilurin contains a dioxane ring fused to the aromatic nucleus, similar to strobilurins E (**3**) (Weber *et al.*, 1990; Bertram *et al.*, 1996) and M (**4**) (Daferner *et al.*, 1998). Correlations in an HMBC experiment between one of the methylene protons (δ_H 4.27) and both C-2' (δ_C 96.4) and C-3 (δ_C 141.7) confirmed this deduction. From here the structure for strobilurin N (**1**) was easily 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 (δ_C 96.4). The HMBC correlation between one of the isopropyl methyls (δ_H 1.37) and the hemiacetalic carbon made this clear and the molecular formula, $C_{21}H_{26}O_7$, deduced by HRMS, with a strong molecular ion m/z 390 $[M]^+$ (100%) indicated that hydroxyl groups are attached to both of the quaternary oxygenated carbons (δ_C 73.7, 96.4). The relative orientation of attachment of the dioxane ring to the benzene nucleus was assumed to be the same as that of strobilurins E (**3**) and M (**4**). These were established from analysis of the fully coupled ^{13}C NMR spectrum and long range selective decoupling experiments. Strobilurin E has also been obtained by total synthesis (Bertram *et al.*, 1996). As expected, measurement of the CD curve of **2** showed no effect 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 (*Bacillus brevis*, *B. subtilis*, *Enterobacter dissolvans*,

Table I. 1H and ^{13}C NMR data for strobilurin N (**1**)^{a,b} (in $CDCl_3$).

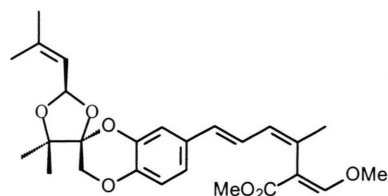
Site	$^{13}C^c$	$^1H^d$	Site	$^{13}C^c$	$^1H^d$
1	114.8 d	6.93 (s)	12	158.9 d	7.43 (s)
2	141.5 s		13	167.9 s	
3	141.7 s		14	23.6 q	1.96 (br. s)
4	117.1 d	6.87 (s)	15	61.9 q	3.85 (s)
5	120.6 d	6.87 (s)	16	51.6 q	3.74 (s)
6	132.7 s		1'	66.3 t	4.00 (d, 11.0)
7	130.4 d	6.38 (d, 16.0)			4.27 (d, 11.0)
8	125.5 d	6.48 (dd, 16.0, 10.5)	2'	96.4 s	
9	129.7 d	6.23 (br. d, 10.5)	3'	73.7 s	
10	130.8 s		4'/5'	23.3 q	1.37 (s)
11	110.9 s			24.2 q	1.41 (s)

^a Numbers in parentheses are coupling constants (*J*) in Hz.

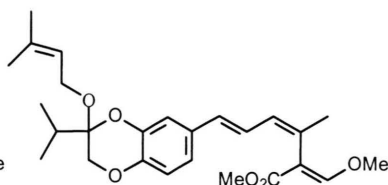
^b Assignments confirmed by two-dimensional experiments (COSY, HMQC, HMBC and NOESY).

^c $CDCl_3$ signal at δ_C 77.0 as reference.

^d $CHCl_3$ signal at δ_H 7.26 as reference.



3 (relative configuration)



4

Micrococcus luteus) or antifungal (*Nematosporea 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 a (*E*)- β -methoxy acrylate unit that shows no anti-fungal 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 spectroscopy showed that this corresponds to the molecular formula $C_{10}H_6O_5$. 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 ^{13}C NMR spectrum (Table II) corroborated these assignments and, furthermore, identified two carbonyls [δ_C 163.8 (br. s) 166.7 (s)] and three quaternary sp^2 -hybridised carbons; all together resonances were observed from ten carbon atoms. The IR spectrum (KBr) is in agreement with the presence of a hydroxyl ($\tilde{\nu}$ 3429 cm^{-1}), an unsaturated lactone carbonyl ($\tilde{\nu}$ 1707 cm^{-1}) and an aromatic acid ($\tilde{\nu}$ 1683 cm^{-1}). It follows from the above data that carboxylic acid **5** is the lactone of dehydrochorismic acid [Kobayashi *et al.*, 1982]. Dehydrochorismic acid

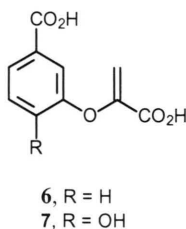
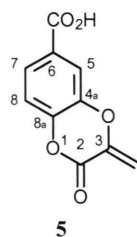


Table II. 1H and ^{13}C NMR data for lactone **5**^{a,b} (in $[D_6]DMSO$).

Site	$^{13}C^c$	$^1H^d$
2	163.8 br. s	
3	151.9 br. s	
4a	142.8 s	
5	121.6 d	7.45 (d, 1.3)
6	121.8 s	
7	127.2 d	7.62 (dd, 8.4, 1.3)
8	116.9 d	6.97 (d, 8.4)
8a	153.3 s	
COOH	166.7 s	
=CH ₂	102.5 t	4.78 (br. s) 5.52 (br. s)

^a Numbers in parentheses are coupling constants (*J*) in Hz.

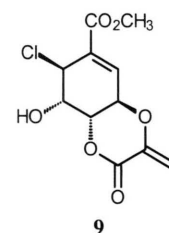
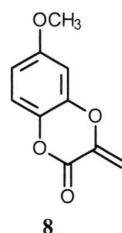
^b Assignments confirmed by two-dimensional experiments (COSY, HMQC and HMBC).

^c $\overline{CHD_2}SOCD_3$ signal at δ_C 39.5 as reference.

^d $\overline{CHD_2}SOCD_3$ signal at δ_H 2.50 as reference.

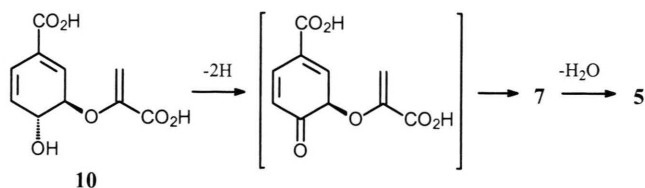
(**7**) has been isolated before from the pollen of *Pinus densiflora* (Kobayashi *et al.*, 1982).

Interestingly, the shikimate-related 3-methylene-benzo[1,4]dioxin-2-one derivatives **8** (Asante *et al.*, 1981) and **9** (Kitamura *et al.*, 1990) have also been reported from fungi.



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

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