ANTIBIOTICS FROM BASIDIOMYCETES. XVIII[1]

STROBILURIN C AND OUDEMANSIN B, TWO NEW ANTIFUNGAL METABOLITES FROM *XERULA* SPECIES (AGARICALES)

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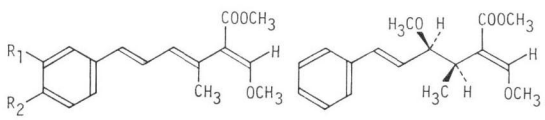
Two new antifungal (*E*)-β-methoxyacrylates, strobilurin C and oudemansin B, were isolated from cultures of *Xerula longipes* and *Xerula melanotricha*. Their structures were elucidated by spectroscopic methods. Both antibiotics inhibit the growth of a wide variety of saprophytic and phytopathogenic fungi at very low concentrations. Like strobilurins A, B, and oudemansin A the new metabolites are potent inhibitors of respiration.

Strobilurins A and B (**1**, **2**)[2,3,4] and oudemansin (**3**)[5] constitute an interesting group of antifungal antibiotics, the biological activity of which depends on the presence of an (*E*)-β-methoxyacrylate moiety[6,7]. These compounds are produced by mycelial cultures of a number of basidiomycetes from the genera Strobilurus, Mycena, Oudemansiella, Hydropus and Cyphellopsis[8,9]. In the following we describe the isolation and structural elucidation of two new members of this group from cultures of *Xerula* R. Mre., a genus closely related to Oudemansiella[9].



1 $R_1 = H$, $R_2 = H$
2 $R_1 = CH_3O$, $R_2 = Cl$
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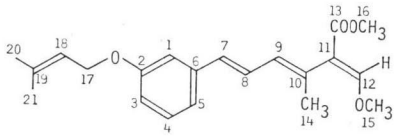
Results and Discussion

Extraction of mycelial surface cultures of *Xerula longipes* (Bull. per St.-Amans) R. Mre. with methanol and isolation of the active components by column chromatography on silica gel followed by preparative TLC on silica gel yielded two main fractions. One was identical with strobilurin B (**2**), the other one was a mixture of a new compound, strobilurin C, and 9-decenoic acid and decanoic acid. The acids were separated by extraction with aqueous sodium hydrogencarbonate and identified by NMR spectroscopy and GC/MS of the corresponding methyl esters.*

Strobilurin C, $C_{21}H_{26}O_4$, exhibits in its ^1H NMR spectrum all signals of the strobilurin side chain[3]. Additional signals for a 1,3-disubstituted benzene ring and a 3,3-dimethylallyloxy residue lead to structure **4**, which is further supported by the MS data. Besides the molecular ion *m/z* 342 strong fragment ions are visible at *m/z* 274 ($M^+ - C_5H_8$), 242 ($M^+ - C_5H_8 - CH_3OH$), 215 ($M^+ - C_5H_8 - CO_2CH_3$), 75 ($C_3H_7O_2$)[3] and 69 (C_5H_9, base peak).

* We thank Prof. Dr. A. PROX, Biberach/Riß, for the GC/MS analysis.



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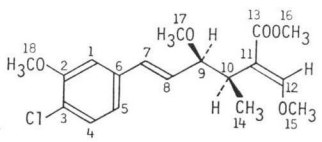

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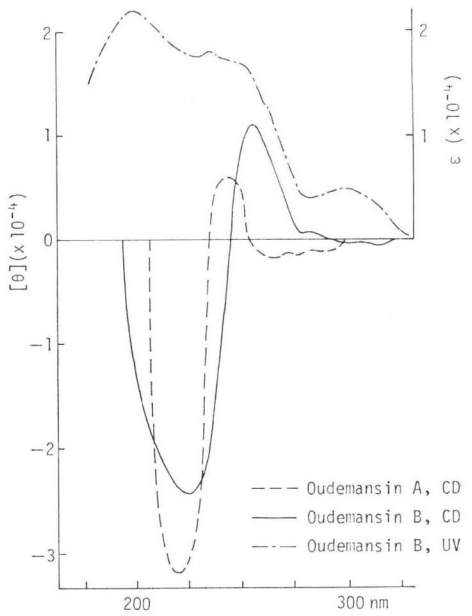
On similar work-up surface cultures of *Xerula melanotricha* Dörfelt afforded besides two yellow pigments[10] and strobilurins A and B (**1**, **2**) a new antifungal compound, oudemansin B. The latter is an optically active oil with the molecular formula $C_{18}H_{23}ClO_5$, suggesting a close relationship to strobilurin B (**2**). Formula **5** is derived for this compound from a comparison of its ^1H and ^{13}C NMR data with those of **2**[8] and **3**[5], which proof the presence of a 3-methoxy-4-chlorophenyl residue and an oudemansin side chain. Confirmation of the aromatic substitution pattern was obtained by ozonolysis which gave 3-methoxy-4-chlorobenzaldehyde[11]. In the MS the typical α-cleavage at the aliphatic methoxy group[5] results in a base ion m/z 211 ($C_{11}H_{12}ClO_2$) and a fragment ion m/z 143 ($C_7H_{11}O_3$). The other fragments are also comparable to those reported for oudemansin. The complete agreement of chemical shifts and coupling constants for oudemansin B and **3** proofs the same relative configuration at the chiral centers for both compounds. A comparison of the CD curves shows a positive Cotton effect in both cases (Fig. 1). Oudemansin B and **3** must therefore possess the same absolute configuration, which, however, is still unknown. In agreement with the designation of the strobilurins A and B, oudemansin (**3**)[5] should be renamed oudemansin A.

Fig. 1. UV and CD spectra of oudemansin B and CD spectrum of oudemansin A in dioxane.



As compared to surface cultures growth and antibiotic production of *X. melanotricha* proceeds much more rapidly in submerged fermentations. A typical time course is shown in Fig. 2. When biotin - aneurin - folic acid (BAF) medium was used, strobilurins A, B as well as oudemansins A and B could be isolated from the culture. In their antibiotic activity both strobilurin C and oudemansin B closely resemble the other naturally occurring (*E*)-β-methoxyacrylates. The new antibiotics inhibit a broad spectrum of different fungi at very low concentrations (Table 1) and are potent inhibitors of eucaryotic respiration. The oxygen uptake of germinating *Penicillium notatum* spores is completely blocked at concentrations of 10^{-6} M strobilurin C and oudemansin B.

Experimental

X. longipes and *X. melanotricha*

Mycelial cultures of *X. longipes* and *X. melanotricha* were obtained from spore prints or tissue plugs

Fig. 2. Time course of a fermentation of *X. melanotricha*.
Dry weight of the mycelium, pH, antifungal activity of the medium in the plate diffusion assay on test plates seeded with spores of *Mucor miehei* (diameter inhibition zone).

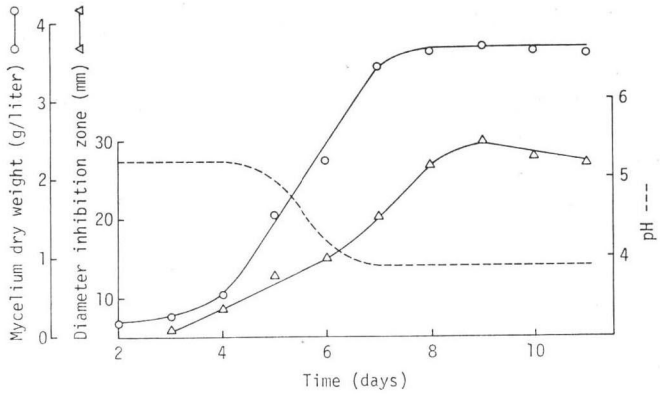


Table 1. Antimicrobial spectra of oudemansin B and strobilurin C (plate diffusion assay).

Test organism	Diameter inhibition zone (mm) μg/disc				
	Oudemansin B			Strobilurin C	
	0.1	1	10	1	10
Absidia glauca CBS 101.08	—	19	22	—	18
A. glauca CBS 102.08	—	23	28	—	22
Alternaria porri	—	18	22	25	35
Ascochyta pisi CBS 126.54	9	20	28	16	25
Ceratocystis retusi CBS 100.78	—	11	40	30	42
Cladosporium cladosporioides	16	27	55	40	55
Mucor miehei	25	32	41	20	27
Nematospora coryli	9	21	38	15	21
Paecilomyces variotii	25	32	36	11	14
Penicillium notatum	23	35	40	16	22
Phytophthora infestans CBS 366.51	26	35	53	20	33
Pleospora herbarum CBS 714.68	20	30	40	—	30
Pythium debaryanum CBS 265.38	10	20	24	—	11
Ustilago nuda CBS 118.19	15	22	50	18	21
Zygorhynchus moelleri CBS 111.10	11	21	27	16	22

from specimen collected in different parts of Germany.

Culture Conditions

Surface cultures were grown in 1 liter Erlenmeyer flasks containing 250 ml of Moser b medium[12] at 20°C for five (*X. longipes*) or seven weeks (*X. melanotricha*). Fermentations were carried out in a Biolafitte fermentation apparatus containing 20 liters of BAF medium[12]. For inoculum 300 ml of seed culture grown in the same medium on a rotary shaker for 12 days at 22°C were used. After inoculation and addition of 1 ml of silicone antifoam the fermentation was conducted at 22°C with agitation (200 rpm for the first five days, then 250 rpm) and aeration (1 liter/minute for the first five days, then 2 liters/minute). Antibiotic production was followed by paper-disc/agar diffusion assay using *Mucor miehei* as test organism.

Isolation of Strobilurin C

Strobilurin C was purified from *X. longipes* mycelia collected from 14 flasks (3.5 liters). The mycelia were extracted with 3 liters of acetone. After evaporation of the solvent strobilurin C was ex-

tracted from the oily residue (0.9 g) with 8 times 20 ml of methylene chloride. The combined extracts were evaporated to dryness, applied to a column (3×30 cm) with silica gel F 60 (Merck) and eluted with cyclohexane - ethyl acetate, 3: 1. The fractions containing strobilurins B and C were pooled and subjected to thin-layer chromatography (silica gel Merck $F60_{254+366}$) using cyclohexane - ethyl acetate - formic acid, 24: 8: 1 as solvent system. Elution of the zone with Rf 0.46 yielded 52 mg of strobilurin B (**2**) which was identified by comparison of its MS and ^1H NMR spectra with those of an authentical sample. The zone with Rf 0.51 contained a mixture of strobilurin C (**4**), decanoic acid and 9-decenoic acid. The mixture was dissolved in ethyl acetate and the acids extracted into a saturated aqueous solution of $NaHCO_3$. After acidification of the aqueous phase, the fatty acids were extracted with ethyl acetate and the solvent evaporated (yield 40 mg). After removal of the fatty acids, strobilurin C was obtained as a homogeneous oil (25 mg).

Strobilurin C

Strobilurin C (**4**), yield 25 mg, clear oil, Rf 0.51, (cyclohexane - ethyl acetate - formic acid, 24: 8: 1), 0.85 (benzene - acetone - ethyl acetate, 7: 3: 1). UV $\lambda_{max}^{CH_3OH}$ (log ε): 297 (4.92), 262 (4.65, sh), 240 (4.86, sh), 231 (4.90, sh), and 226 nm (4.92).

IR ($CHCl_3$): 3040 (w), 2955 (st), 2870 (w), 1708 (sst), 1630 (m), 1594 (w), 1580 (sh), 1486 (w), 1450 (m, br), 384 (m), 1289 (sh), 1245 (st), 1165 (m), 1150 (m), 1127 (sst), 1080 (m), 1015 (m, br), 968 (m), 887 (w), and 687 cm^{-1} (w).

^1H NMR (400 MHz, $CDCl_3$, TMS as internal standard): δ 1.75 (br. s, 20-H), 1.80 (br. s, 21-H), 1.97 (d, 14-H), 3.73 (s, 16-H), 3.84 (s, 15-H), 4.51 (d, 17-H), 5.49 (tqq, 18-H), 6.25 (dd, 9-H), 6.45 (d, 7-H), 6.60 (dd, 8-H), 6.76 (m, 3-H), 6.91 (m, 1-H), 6.93 (m, 5-H), 7.19 (dd, 4-H), and 7.43 (s, 12-H); $J_{3,4}=J_{4,5}=8$, $J_{7,8}=15.6$, $J_{8,9}=10.8$, $J_{9,14}=1.4$, $J_{17,18}=6.8$ and $J_{18,20}=J_{18,21}=1.2$ Hz.

MS (AEI-MS50, 150°C, direct probe insert): *m/z* 342.1824 (M^+, 33.6%, calcd. $C_{21}H_{26}O_4$ 342.1831), 274 (23%, $C_{16}H_{18}O_4$), 243 (16.3, $C_{15}H_{15}O_3$), 242 (72.9, $C_{15}H_{14}O_3$), 227 (22.6, $C_{14}H_{11}O_3$), 215 (37.5, $C_{14}H_{15}O_2$), 210 (15.5, $C_{14}H_{10}O_2$), 183 (58.1, $C_{13}H_{11}O$), 182 (20.7, $C_{13}H_{10}O$), 167 (7.6, $C_9H_{11}O_3$), 158 (44.5, $C_{11}H_{10}O$), 143 (13.8, $C_7H_{11}O_3$), 137 (61.2, $C_8H_9O_2$), 121 (18.3, $C_7H_5O_2$), 85 (19.3, $C_4H_5O_2$), 84 (4.1, C_5H_8O), 75 (60.6, $C_3H_7O_2$), 69 (100, C_5H_9), 68 (13.5, C_5H_8), and 41 (81.9, C_3H_5).

Decanoic Acid and 9-Decenoic Acid

For esterification[13], the fatty acid mixture (40 mg) and 5 mg of (4-dimethylamino)pyridine were dissolved in 5 ml of methylene chloride and 3 ml of methanol. 60 mg of dicyclohexylcarbodiimide were added with stirring at 0°C and the mixture was allowed to stand for 1 hour at 0°C and 6 hours at 20°C. The solution was evaporated to dryness after filtration. GC/MS analysis yielded two components *m/z* 186 and *m/z* 184. The identity of *m/z* 186 with decanoic acid was established by comparison of the mass spectra. The position of the double bond in *m/z* 184 was deduced from the ^1H NMR spectrum (400 MHz, $CDCl_3$) of the fatty acid mixture exhibiting typical signals for the vinyl group at δ 5.81 (m, 1H), 4.99 (br. d, J=17 Hz, 1H), and 4.93 (br. d, J=10 Hz, 1H).

The ratio of 9-decenoic acid - decanoic acid, 6: 4 in the mixture, was determined by integrating the triplet for the 2-CH_2 groups at δ 2.35 and the singlet for the 9-decenoic acid at δ 5.81.

Isolation of Oudemansin B

From Surface Cultures: Strobilurins A, B and oudemansin B were purified from the acetone extract of *X. melanotricha* mycelia collected from 16 flasks (4 liters). The crude product (1.8 g) was purified by column chromatography and PTLC on silica gel as described above for strobilurin C. Yields: Strobilurin A, 8 mg; strobilurin B, 12 mg; oudemansin B, 34 mg.

From fermentations: After removal of the mycelia by filtration the culture filtrate (18 liters) was extracted with 5 liters of ethyl acetate, the organic phase yielding 0.62 g of crude product. This was applied to several successive columns (2.5×10 cm) with silica gel (Mallinckrodt), which were eluted with chloroform. Final purification of the strobilurins and oudemansins was achieved by PTLC as described above. Yields: Strobilurin A, 19 mg; strobilurin B, 11 mg; oudemansin A, 105 mg; oudemansin B, 35 mg. Approximately equal amounts of the individual antibiotics were obtained from the crude extract obtained by extraction of the lyophilized mycelia with acetone.

Oudemansin B

Yellowish oil, $[\alpha]_D^{22}$ −8.3° (*c* 1.52, $CHCl_3$); Rf 0.42 (cyclohexane - ethyl acetate - formic acid, 24: 8: 1).

UV $\lambda_{max}^{dioxane}$ (log ε): 312 (3.49), 299 (3.67), 270 (4.11, sh), 261 (4.22), 248 (4.25), 219 (4.34) and 203nm (4.19, sh). CD (dioxane): $[\theta]_{319.5}=0$; $[\theta]_{311.5}=-0.79\times10^3$; $[\theta]_{302}=-0.53\times10^3$; $[\theta]_{293.5}=0$; $[\theta]_{284.5}=+0.59\times10^3$; $[\theta]_{280}=+1.35\times10^3$; $[\theta]_{264.5}=+10.99\times10^3$; $[\theta]_{255.5}=0$; $[\theta]_{239}=-24.42\times10^3$; $[\theta]_{214.5}=0$.

IR ($CHCl_3$): 3050 (m), 2960 (m), 2900 (w), 2870 (w), 2845 (w), 1708 (sst), 1649 (st), 1600 (m), 1580 (m), 1497 (m), 1469 (m), 1440 (w), 1415 (m), 1332 (w), 1285 (st), 1245 (st, sh), 1145 (st), 1125 (st), 1085 (st), 1070 (st), 1038 (m), 1000 (w), 970 (m), 915 (w), 865 (w), 535 (w, br.), and 480 cm^{-1} (w).

^1H NMR (90 MHz, CD_3OD, TMS internal standard): δ 1.21 (d, 14-H), 2.95 (dq, 10-H), 3.29 (s, 17-H), 3.60 (s, 16-H), 3.79, 3.85 (both s, 15- or 18-H), 3.98 (dd, 9-H), 5.88 (dd, 8-H), 6.42 (d, 7-H), 6.88 (ddd, 5-H), 6.99 (d, 1-H), 7.22 (d, 4-H), and 7.30 (s, 12-H); $J_{1,5}=2$, $J_{4,5}=8$, $J_{5,7}=0.3$, $J_{7,8}=16$, $J_{8,9}=8.4$, $J_{9,10}=9.6$, and $J_{10,14}=7$ Hz.

^{13}C NMR ($^{12}CDCl_3$, TMS internal standard): δ 15.7 (q, C-14), 35.7 (d, C-10), 51.0 (q, C-16), 56.1, 56.7 (both q, C-17 or C-18), 64.4 (q, C-15), 84.9 (d, C-9), 109.9 (d, C-1), 112.3 (s, C-11), 119.5 (d, C-5), 121.5 (s, C-3), 130.2 (d, C-4), 130.5 (d, C-8), 131.5 (d, C-7), 137.1 (s, C-6), 155.1 (s, C-2), 159.6 (d, C-12), and 168.3 (s, C-13).

MS (AEI-MS50, 150°C, direct probe insert): *m/z* 354.1226 (M^+, 1.3%, calcd. $C_{18}H_{23}{}^{35}ClO_5$ 354.1234), 322 (0.4, $C_{17}H_{19}ClO_4$), 291 (1.0, $C_{16}H_{16}ClO_3$), 211 (100, $C_{11}H_{12}ClO_2$), 179 (13.8, $C_{10}H_8ClO$), 143 (12.5, $C_7H_{11}O_3$), and 75 (16.7, $C_3H_7O_2$).

Ozonolysis of Oudemansin B

25 mg of oudemansin B were dissolved in 10 ml of methanol and cooled to −30°C. Within 10 minutes approximately 100 mg of ozone were passed through the solution while the temperature was lowered to −60°C. Then, after removal of excess ozone by bubbling nitrogen through the reaction mixture for 5 minutes, 0.2 ml of dimethyl sulfide were added and the solution stirred for 1 hour at −10°C, 1 hour at 0°C, and 1 hour at room temperature. The solvent was removed under reduced pressure, then water was added to the residue and the aqueous phase extracted several times with ethyl acetate. The combined extracts were evaporated to dryness and the residue subjected to PTLC using cyclohexane - ethyl acetate - formic acid (24: 8: 1) as solvent system. Elution of the zone with Rf 0.54 yielded 6.4 mg of 4-chloro-3-methoxybenzaldehyde, colorless crystals, mp 50~51°C (Ref.[11] 52°C); ^1H NMR (90 MHz, $CDCl_3$, TMS internal standard); δ 3.94 (s, 3H), 7.3~7.7 (m, 3H), and 9.95 (s, 1H); MS (AEI-MS50, 50°C, direct probe insert): *m/z* 170.0134 (M^+, 14.23% calcd. 170.0134).

Biological Assays

The antimicrobial spectra and inhibition of respiration of *Penicillium notatum* were evaluated as described previously[2,5].

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References

1) KUPKA, J.; T. ANKE, K. MIZUMOTO, B.-M. GIANNETTI & W. STEGLICH: Antibiotics from basidiomycetes. XVII. The effect of marasmic acid on nucleic acid metabolism. J. Antibiotics 36: 155~160, 1983
2) ANKE, T.; F. OBERWINKLER, W. STEGLICH & G. SCHRAMM: The strobilurins, new antifungal antibiotics from the basidiomycete *Strobilurus tenacellus* (Pers. ex Fr.) Sing. J. Antibiotics 30: 806~810, 1977
3) SCHRAMM, G.; W. STEGLICH, T. ANKE & F. OBERWINKLER: Antibiotika aus Basidiomyceten. III. Strobilurin A und B, antifungische Stoffwechselprodukte aus *Strobilurus tenacellus*. Chem. Ber. 111: 2779~2784, 1978
4) Compare also: SEDMERA, P.; V. MUSILEK, F. NERUD & M. VONDRACEK: Mucidin: Its identity with strobilurin A. J. Antibiotics 34: 1069, 1981

5) ANKE, T.; H.-J. HECHT, G. SCHRAMM & W. STEGLICH: Antibiotics from basidiomycetes. IX. Oudemansin, an antifungal antibiotic from *Oudemansiella mucida* (Schrader ex Fr.) Hoehnel (Agaricales). J. Antibiotics 32: 1112~1117, 1979
6) SCHRAMM, G.; W. STEGLICH & T. ANKE: Structure-activity relationship of strobilurins, oudemansin, and synthetic analogues. Abstracts XIII International Congress of Microbiology, p. 153. Boston, 1982
7) BECKER, W. F.; G. VON JAGOW, T. ANKE & W. STEGLICH: Oudemansin, strobilurin A, strobilurin B, and myxothiazol: New inhibitors of the bc_1 segment of the respiratory chain with an E-β-methoxyacrylate system as common structural element. FEBS Lett. 132: 329~333, 1981
8) BÄUERLE, J.: Antibiotika aus Basidiomyceten der Gattungen *Clitopilus*, *Hohenbuehelia*, *Hemimycena* und *Mycena* (Agaricales). Dissertation, University of Tübingen, 1981
9) DÖRFELT, H.: Taxonomische Studien in der Gattung *Xerula* R. Mre. Feddes Repert. 90: 363~388, 1979
10) HERRMANN, R.: Untersuchungen zur Konstitution, Synthese und Biosynthese von Pilzfarbstoffen. Dissertation, University of Bonn, 1980
11) THALLER, V. & J. L. TURNER: Natural acetylenes. XXXV. Polyacetylenic acid and benzenoid metabolites from cultures of the fungus *Lepista diemii* Singer. J. Chem. Soc. Perk. Trans. I 1972: 2032~2034, 1972
12) MOSER, M.: Die Gattung *Phlegmacium* (Schleimköpfe). p. 59, J. Klinkhardt, Bad Heilbrunn, 1960
13) NEISES, B. & W. STEGLICH: Einfaches Verfahren zur Veresterung von Carbonsäuren. Angew. Chem. Internat. Edit. Engl. 90: 556~557, 1978