VOL. 53 NO. 3, MAR. 2000 THE JOURNAL OF ANTIBIOTICS pp. 297 — 300

New Strobilurins O and P from a Mushroom

NOBUO HOSOKAWA, ISAO MOMOSE, RYUICHI SEKIZAWA, HIROSHI NAGANAWA, HIRONOBU IINUMA and TOMIO TAKEUCHI

Institute of Microbial Chemistry, 3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141-0021, Japan

SUSUMU MATSUI

TAKARA AGRI Co., Ltd., 2257 Noji-cho, Kusatsu-City, Shiga 525-0055, Japan

(Received for publication November 24, 1999)

During screening for nematocidal activities, *Mycena galericulata* (Scop.: Fv.) S. F. Grey was found to produce toxins active against *Caenorhabditis elegans*. Two new strobilurins O (**1**) and P (**2**) were isolated from the mycelia using EtOAc extraction, silica gel column chromatography and preparative silica gel TLC monitoring of nematocidal activity. In this report we describe fermentation, physico-chemical properties, biological activities and structures of the new strobilurins.

Nematocidal activity was determined in a 24-well plate assay using the free-living nematode, *C. elegans*. In the screening, we used MeOH extracts of the mushroom mycelia mixed with 1.7% agar medium, with the concentration of MeOH adjusted to 1%. Worms after L1 stage were placed at 20~30 in number in each of the wells of an agar plate and incubated at 20°C for 36 hours. Nematocidal activity was observed under the microscope. Milbemycin was used as the standard nematocidal substance.

From an agar slant culture of *M. mycena*, mycelia of 5 mm squae together with agar were taken and inoculated into 500-ml Erlenmeyer flasks each of which contained 110 ml of a medium composed of 1.0% glucose, 0.5% peptone, 0.3% yeast extract, 0.3% KH_2PO_4 and 0.1%

Table 1. Physico-chemical properties of strobilurins O (**1**) and P (**2**).

	1	**2**
Appearance	Colorless oil	Colorless oil
Solubility	Soluble: $CHCl_3$, EtOAc, MeOH Insoluble: H_2O	Soluble: $CHCl_3$, EtOAc, MeOH Insoluble: H_2O
Molecular formula	$C_{26}H_{34}O_7$	$C_{26}H_{32}O_7$
FAB-MS	458	456
UV λmax nm (log ε) (MeOH)	230 (4.36), 235 (sh)(4.36), 290 (sh)(5.64), 300 (4.46), 320 (sh)(4.69)	225 (4.36), 290 (sh)(4.19), 300 (4.23), 320 (sh)(4.21)
IRυmax cm^{-1} ($CHCl_3$)	3005, 2960, 1720, 1640, 1580, 1520, 1470, 1450, 1280, 1250, 1160, 1135, 1090	2995, 2950, 1700, 1640, 1620, 1500, 1440, 1380, 1260, 1220, 1160, 1100
$[\alpha]^{22}_D$	-55° (*c* 0.18, MeOH)	-36° (*c* 0.23, MeOH)
Rf-value Silica gel (TLC) (Hexane:EtOAc=2:1)	0.31	0.53
HPLC retention time (min)*	9	16

*ODS (4.6 x 150 mm), 60% CH_3CN, 2 ml/min, 250 nm

Table 2. ^{13}C and ^{1}H NMR shifts of strobilurins O (**1**) and P (**2**).

Carbon No.	**1** δ_C ppm (125 MHz)	**1** δ_H pm (J in Hz, 500 MHz)	**2** δ_C ppm (125 MHz)	**2** δ_H ppm (J in Hz, 500 MHz)
1	122.4 (d)	6.95 (1H, d, 2.1)	121.3 (d)	6.96 (1H, d, 2.1)
2	146.7 (s)	—	146.8 (s)	—
3	150.6 (s)	—	150.6 (s)	—
4	120.7 (d)	6.85 (1H, d, 7.9)	120.9 (d)	6.86 (1H, d, 7.9)
5	121.5 (d)	6.93 (1H, dd, 7.9, 2.1)	122.4 (d)	6.94 (1H, dd, 2.1, 7.9)
6	133.8 (s)	—	134.0 (s)	—
7	130.3 (d)	6.37 (1H, brd, 15.6)	130.3 (d)	6.38 (1H, brd, 15.6)
8	125.7 (d)	6.48 (1H, dd, 10.7, 15.6)	125.9 (d)	6.49 (1H, dd, 11.0, 15.6)
9	129.8 (d)	6.22 (1H, brd, 10.7, 1.2)	129.7 (d)	6.23 (1H, q, 1.5, 11.0)
10	130.9 (s)	—	131.0 (s)	—
11	110.8 (s)	—	110.8 (s)	—
12	158.9 (d)	7.43 (1H, s)	158.9 (d)	7.43 (1H, s)
13	167.8 (s)	—	167.8 (s)	—
14	23.7 (q)	1.96 (3H, brs)	23.7 (q)	1.96 (3H, brs)
15	61.9 (q)	3.84 (3H, s)	61.9 (q)	3.85 (3H, s)
16	51.6 (q)	3.74 (3H, s)	51.6 (q)	3.74 (3H, s)
17	68.1 (t)	4.22 (1H, dd, 2.8, 12.8)	69.4 (t)	4.25 (1H, dd, 2.8, 13.1)
		4.04 (1H, dd, 7.0, 12.8)		4.13 (1H, dd, 5.2, 13.1)
18	83.5 (d)	3.57 (1H, dd, 2.8, 7.0)	75.2 (d)	5.08 (1H, dd, 2.8, 5.2)
19	80.5 (s)	—	79.5 (s)	—
20	27.5 (q)	1.48 (3H, s)	26.4 (q)	1.38 (3H, s)
21	21.4 (q)	1.29 (3H, s)	23.8 (q)	1.37 (3H, s)
22	69.6 (t)	3.38 (1H, dd, 6.4, 11.0)	165.6 (s)	—
		3.88 (1H, dd, 4.6, 11.0)		
23	62.1 (d)	2.98 (1H, dd, 4.6, 6.4)	115.6 (d)	5.79 (1H, m)
24	57.6 (s)	—	158.6 (s)	—
25	24.7 (q)	1.35 (3H, s)	27.5 (q)	1.92 (3H, d, 1.4)
26	18.9 (q)	1.30 (3H, s)	20.4 (q)	2.19 (3H, d, 1.4)

$MgSO_4 \cdot 7H_20$, and stationary cultured for 13 days at 27°C, then the incubation was continued for 9 more days on a rotary shaker.

The mycelia (270 g, wet) were collected on a filter and extracted with MeOH (3.0 liters×2). The extract was concentrated to a volume of 100 ml under reduced pressure. Two liters each of H_2O and EtOAc were added to the concentrated extract, and the active substance was extracted into EtOAc. The EtOAc layer was separated and then evaporated under reduced pressure to give an oily residue (1.3 g). The oily residue was applied to a silica gel column (5×30 cm equilibrated with hexane) and the active fractions eluted with hexane : EtOAc (2 : 1) were collected and concentrated under reduced pressure to give another oily substance. This substance was further purified using preparative TLC developed with hexane : EtOAc=2 : 1 to

give 20 mg of **1** and 23 mg of **2**. Other strobilurins, G and I[1,2], were also produced by this mushroom.

Physico-chemical properties of **1** and **2** are summarized in Table 1. The molecular formula of **1** was determined to be $C_{26}H_{34}O_7$ (MW 458) using FAB-MS (*m/z*; 458) and NMR. UV absorption maxima and its intensities showed the presence of a triene structure in the new strobilurins. The ^1H and ^{13}C NMR spectra of **1** were very similar to those of strobilurin G[3] except for the NMR data around 23 and 24 positions (Table 2). The ^{13}C signals of **1** and **2** at the 23 and 24 positions were observed at 62.1 and 57.6 ppm, respectively. The ^{13}C-^1H spin coupling constant of C-23 was 170 Hz indicating the presence of an oxirane ring as shown in Fig. 2. Based on these results, the structure of **1** was determined to be 23,24-epoxystrobilurin G (strobilurin O) as shown in Fig. 1.

The molecular formula of **2** was determined to be $C_{26}H_{32}O_7$ (MW 456) by FAB-MS and NMR. In the ^{13}C NMR spectrum of **2**, the methylene signal of C-22 (69.55 ppm), which was observed in the spectrum of **1**, disappeared, and one carbonyl carbon was observed at 165.6 ppm. In the HMBC spectrum of **2**, a long-range coupling was observed between an olefin proton at H-23 (5.79 ppm) and the carbonyl carbon (C-22) as shown in Fig. 2. This observation and IR absorption band at 1700 cm^{-1} were consistent with the presence of a 3-methylcrotonate

Fig. 1. Structures of strobilurins O (**1**) and P (**2**).

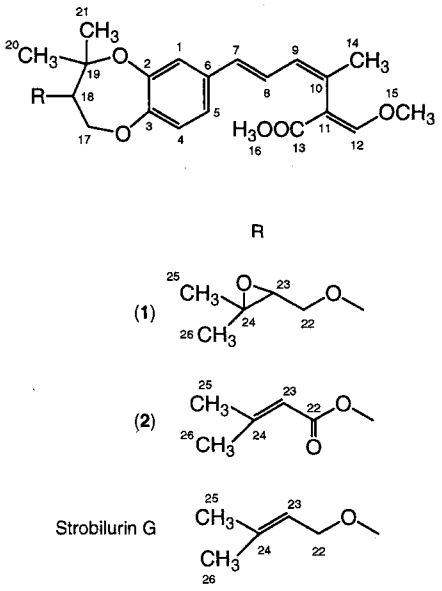


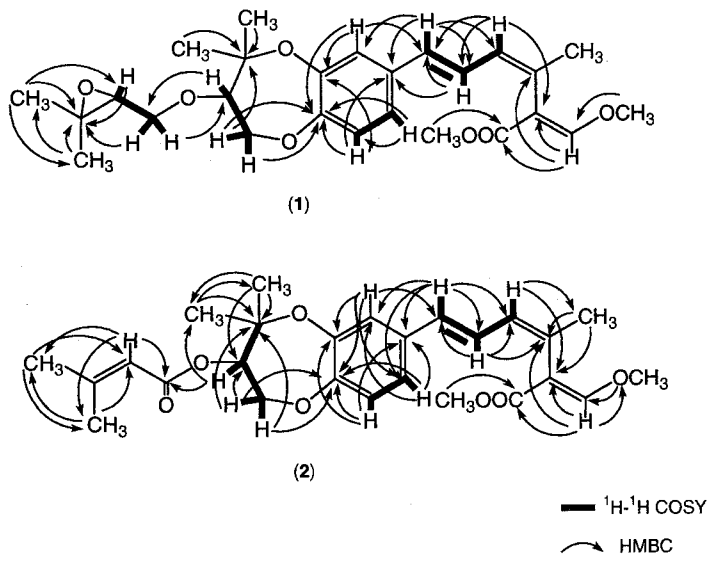
Table 3. Nematocidal activities of **1**, **2**, strobilurin G and milbemycin against *Caenorhabditis elegans*.

Compounds	ND_{90}* (μg/ml)
1	0.5
2	0.3
Strobilurin G	4.0
Milbemycin	0.05

After L1 stage worms were seeded at 20~30 in number in a 24-well agar plate and were incubated at 20°C for 36 hours.

* Concentration causing more than 90% immotility of *C. elegans*.

Fig. 2. ^1H-^1H COSY and HMBC data on strobilurins O (**1**) and P (**2**).



moiety in **2**. Consequently, the structure of **2** was determined to be 22-oxostrobilurin G (strobilurin P).

Nematocidal activities of **1**, **2** and strobilurin G are shown in Table 3. Compounds **1** and **2** were about 10 times more potent than strobilurin G and both compounds exhibited strong cytotoxicities against HeLa-S3 human cervical carcinoma, similar to strobilurin G (IC_{50}= 0.02~0.06 μg/ml). **1** and **2** also exhibited weak antifungal activities against *Pyricularia oryzae* P-2 (MIC= 12.5 μg/ml) and *Botrytis cinera* B-22 (MIC=25 μg/ml) when determined using the agar dilution method.

Acknowledgement

We thank Hokko-Kagaku Co. Ltd. for the determination of antifungal activities of strobilurins.

References

1) FREDENHAGEN, A.; A. KUHN, H. PETER, V. CUOMO & U. GIVULIANO: Strobilurins F, G and H, three new antifungal metabolites from *Bolinea lutea* I. Fermentation, isolation and biological activity. J. Antibiotics 43: 655~660, 1990
2) HELLWIG, V.; J. DASENBROCK, D. KLOSTERMEYER, S. KROISS, T. SINDLINGER, P. SPITELLER, B. STEFFAN, W. STEGLICH, M. ENGLER-LOHR, S. SEMAR & T. ANKE: New benzodioxepin type strobilurins from *Basidiomycetes*. Structural revision and determination of the absolute configuration of strobilurin D and related β-methoxy-acrylate antibiotics. Tetrahedron 55: 10101~10118, 1999
3) FREDENHAGEN, A.; P. HUG & H. H. PETER: Strobilurins F, G and H, three antifungal metabolites from *Bolinea lutea*. II. Structure determination. J. Antibiotics 43: 661~667, 1990