ANTIBIOTICS FROM BASIDIOMYCETES. XXIX†: PILATIN, A NEW ANTIBIOTICALLY ACTIVE MARASMANE DERIVATIVE FROM CULTURES OF *FLAGELLOSCYPHA PILATII* AGERER

J. HEIM and T. ANKE

LB Biotechnologie der Universität,
Paul-Ehrlich-Str. 23, D-6750 Kaiserslautern, FRG

U. MOCEK, B. STEFFAN and W. STEGLICH

Institut für Organische Chemie und Biochemie der Universität,
Gerhard-Domagk-Str. 1, D-5300 Bonn 1, FRG

(Received for publication June 11, 1988)

Pilatin, a new marasmane derivative, was isolated from fermentations of the cyphelloid fungus *Flagelloscypha pilatii*. Its structure was determined by chemical and physical methods. Pilatin inhibits the growth of bacteria and fungi at concentrations of 5~50 μg/ml. The compound is highly cytotoxic. The incorporation of thymidine and uridine into DNA and RNA in Ehrlich carcinoma ascitic cells is strongly inhibited by pilatin. Like marasmic acid pilatin causes frameshift mutations in *Salmonella typhimurium* TA 98.

Flagelloscypha pilatii is a small astipitate cup shaped basidiomycete growing on grasses. The species was first described by AGERER in 1975[2]. *Flagelloscypha* (tribus *Marasmieae*, family *Tricholomataceae*) is related to the genera *Oudemansiella*, *Strobilurus*, *Marasmius*, and *Crinipellis*, from which a number of antibiotic metabolites have been described previously (for review see ref 3). *F. pilatii* JK 322 was detected as an antibiotic producing strain during a screening of cyphelloid fungi for antimicrobial and cytotoxic compounds. In the following we wish to describe the fermentation, isolation, and biological characterization of pilatin, the first secondary metabolite isolated from a *Flagelloscypha* species.

Materials and Methods

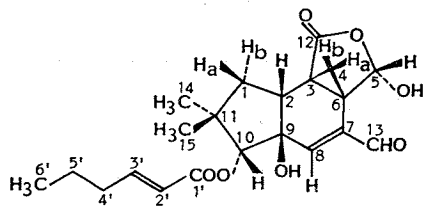
Flagelloscypha pilatii

Mycelial cultures of *F. pilatii* strain JK 322 were obtained from tissue plugs of fruiting bodies collected in Tübingen, FRG. The strain used in this work and herbarium specimen have been deposited in the collection of the Lehrbereich Biotechnologie, University of Kaiserslautern.

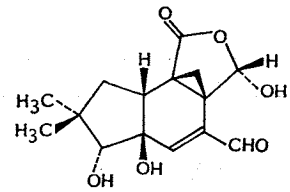
Fermentation and Isolation

For maintenance on agar slants and submerged cultivation the fungus was grown in a yeast extract - malt extract - glucose (YMG) medium composed of (g/liter): Yeast extract 4, malt extract 10, and glucose 4. For the production of pilatin, strain JK 322 was grown in 20 liter of YMG medium in a Biolafitte C-6 fermentor (3 liters air/minute, 120 rpm, 22°C). 200 ml of a well grown seed culture were used as inoculum. Antibiotic production was followed by paper disc-agar diffusion assay using *Bacillus brevis* as test organism. After 8~10 days of fermentation the mycelia were separated from the culture fluid by filtration. Pilatin was extracted from the culture fluid (19 liters) with 5 liters of EtOAc.

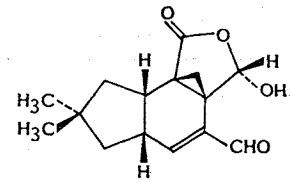
† See ref 1.



1



2



3

After evaporation of the solvent the crude product (3 g) was applied to a column (2.5×10 cm) with silica gel (Mallinckrodt) and eluted with CH_2Cl_2. The pilatin containing fractions (25~51, 10 ml each) were pooled, the solvent evaporated and the product (1 g) applied to a column (2.5×50 cm) of Sephadex LH-20 and eluted with MeOH. Pure pilatin was obtained from fractions 33~39 (fraction volume 15 ml). Yield 500 mg.

Physical and Spectroscopic Data

The NMR spectra were recorded on a Bruker AM-400 spectrometer. Data for the symmetrized INADEQUATE 2D experiment: 240 mg sample in $CDCl_3$; relaxation delay D1=4 seconds, spin-echo period D2=0.003333 second, initial evolution delay DO=3E-6 seconds, NE=128, SW=20,000 Hz, SW1=5,000 Hz. TLC was performed on alumina foils, Silica gel 60 F_{254} Merck, Darmstadt, No. 5554.

Pilatin (1)

Colorless oil; $[\alpha]_D^{20}$ −97.6° (*c* 0.41, $CHCl_3$); Rf 0.46 (C_6H_6 - HCOOEt - HCOOH, 10:5:3); 0.65 (C_6H_6 - Me_2CO - AcOH, 17:2:1); UV λ_{max}^{MeOH} nm (log ε) 211 (4.16), 240 (2.72); CD (MeOH) $[\theta]_{282}=0$, $[\theta]_{330}=+1.99\times10^3$; IR (KBr) cm^{-1} 3400 (st, br), 2940 (st), 2910 (sh,) 2850 (m), 1765 (st), 1745 (st), 1708 (st), 1680 (sst), 1640 (m), 1450 (m), 1360 (w), 1300 (w), 1250 (w), 1215 (m), 1170 (m), 1120 (m), 1080 (m), 1040 (m), 954 (w), 915 (w); high resolution (HR)-MS (direct inlet, 200°C) *m/z* (relative intensity, %) 390.1680 (4.5, M^+, calcd for $C_{21}H_{26}O_7$ 390.1678), 346 (0.3, $C_{19}H_{22}O_6$), 321 (0.9, $C_{17}H_{21}O_6$), 318 (2.1, $C_{17}H_{18}O_6$), 308 (0.4, $C_{16}H_{20}O_6$), 294 (1.7, $C_{15}H_{18}O_6$), 290 (0.6, $C_{16}H_{18}O_5$), 276 (38.7, $C_{15}H_{16}O_5$), 97 (100, C_6H_9O), 55 (76.3, C_3H_3O).

Pilatol (9β,10α-Dihydroxymarasmic Acid, 2)

To **1** (11 mg) in MeOH (3 ml) was added 0.5 N NaOH (0.7 ml). After 5 hours at room temperature the saponification of **1** was complete (TLC control). **2** was isolated in the usual way and purified by chromatography on a small Sephadex LH-20 column (eluant MeOH): Colorless oil; Rf 0.25 (C_6H_6 - HCOOEt - HCOOH, 10:5:3); CD (MeOH) nm $[\theta]\times10^3$: 205 (0), 215 (+29.83), 230 (0), 243 (−22.08), 270 (0), 325 (+2.71), 370 (0); ^1H NMR (400 MHz, MeOH-d_3) δ 0.83 (s, 14-CH_3), 1.14 (s, 15-CH_3), 1.27 (dd, *J*=13.5 and 10.5 Hz, 1-H_a), 1.41 (br m, 4-H_a), 1.64 (d, *J*=4.2 Hz, 4-H_b), 2.06 (dd, *J*=13.5 and 9 Hz, 1-H_b), 2.76 (dd, *J*=10.5 and 9 Hz, 2-H), 3.86 (s, 10-H), 6.06 (br s, 5-H), 6.96 (s, 8-H), 9.64 (s, 13-H); HR-MS (direct inlet, 180°C) *m/z* (relative intensity, %) 294.1079 (0.04, M^+, calcd for $C_{15}H_{18}O_6$ 294.1104), 246 (15), 168 (15), 107 (100).

Biological Assays

The antimicrobial spectra and macromolecular syntheses in cells of the ascitic form of Ehrlich carcinoma were measured as described previously[4,5]. Mutagenicity was tested as described by AMES *et al.*[6]. *Salmonella typhimurium* strains TA 100, TA 98, TA 1535, and TA 1538 were used for the spot test without rat liver microsomes.

Results and Discussion

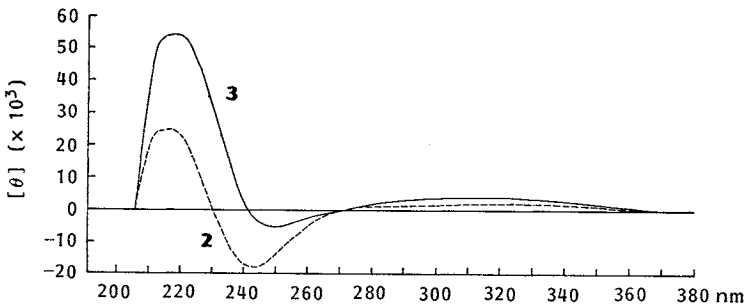
According to the HR-MS pilatin has the molecular formula $C_{21}H_{26}O_7$. In the ^1H NMR spectrum (Table 1) the signals for an aldehyde group, three olefinic protons, two tertiary methyl groups and

Table 1. ^{1}H and ^{13}C NMR data of pilatin (**1**) (400 and 100.6 MHz respectively; $CDCl_3$ as solvent and internal standard).

Proton[a]	δ	J (Hz)	Carbon	δ	J (Hz)
1-H_a	1.27 dd	13.5, 12.0	C-1	42.41 Dm	132.2
1-H_b	2.16 dd	13.5, 8.5			
2-H	2.99 dd	12.0, 8.5	C-2	34.67 Dm	137.0
			C-3	31.54 m	
4-H_a	1.38 d	5.0	C-4	26.44 T	168.3
4-H_b	1.59 d	5.0			
5-H	6.08 br s		C-5	96.91 Dt	181.7, 4
			C-6	30.45 m	
			C-7	139.97 dm	28.4
8-H	6.49 s		C-8	142.45 Dt	158.8, 3.2
			C-9	78.89 m	
10-H	4.91 s		C-10	90.34 Dm	149.3
			C-11	37.14 m	
			C-12	174.61 dd	10,4
13-H	9.52 s		C-13	192.84 Dd	178.8, 8.6
14-CH_3	0.88 s		C-14	25.41 Qm	126.3
15-CH_3	1.16 s		C-15	30.52 Qm	126.3
			C-1′	168.52 m	
2′-H	5.92 dt	15.8, 1.5	C-2′	119.98 Dtd	164.0, 6.0, 14
3′-H	7.08 dt	15.8, 7.0	C-3′	152.67 Dm	154.4
4′-H	2.22 ddt	7.5, 7.0, 1.5	C-4′	34.36 Tm	137.2
5′-H	1.50 septet	7.5	C-5′	21.08 Tm	128.0
6′-CH_3	0.93 t	7.5	C-6′	13.63 Qm	125.0

[a] Protons correlated by NOE difference measurements: 1-H_a/15-CH_3; 1-H_a/2-H; 2-H/4-H_b; 2-H/10-H; 4-H_a/5-H; 8-H/13-H; 10-H/15-CH_3.
D, T, Q, M: Doublet, triplet, quartet, multiplet; the capital letters indicate direct coupling. Small letters (d, t, q, m) indicate long range coupling.

Fig. 1. CD spectra of pilatol (**2**) and marasmic acid (**3**) in MeOH.



an ethyl unit are visible.

By use of the ^{13}C-^{13}C 2D correlation (symmetrized INADEQUATE 2D experiment)[7~9] the connectivities of the carbon atoms in the hexenoyl unit and the ring system with exception of the lactol moiety were determined. By means of a modified ^{13}C-^{1}H long range correlation[10] the attachment of the hexenoyl residue to the hydroxy group at C-10 was secured as well as the annelation of the lactol moiety to the cyclopropane ring. The arrangement of the lactol ring shown in formula **1** was confirmed by selective decoupling of 5-H and 8-H in the ^{1}H-coupled ^{13}C NMR spectrum which led in both cases to a sharpening of the C-6 and C-7 signals. This experiment was carried out at −45°C because

Table 2. MICs of pilatin (**1**) and marasmic acid (**3**).

	MIC (μg/ml)	
	Pilatin	Marasmic acid
Bacteria		
Aerobacter aerogenes	≥50	≥50
Arthrobacter citreus	20	5
Bacillus brevis	5	1
B. subtilis	10	2
Corynebacterium insidiosum	5	5~10
Escherichia coli	≥50	2
Micrococcus roseus	NT	10
Mycobacterium phlei	≥50	10~20
Proteus vulgaris	50	5
Pseudomonas fluorescens	≥50	≥50
Micrococcus luteus	50	10
Staphylococcus aureus	50	5~10
Streptomyces sp. PRL 1642	20	5
Streptomyces sp. ATCC 23836	10	5
Fungi		
Candida albicans	≥50	≥50
Nematospora coryli	10	0.1
Nadsonia fulvescens	50	≥50
Rhodotorula glutinis	≥50	≥50
Saccharomyces cerevisiae	≥50	≥50
S. cerevisiae is 1	≥50	5

NT: Not tested.

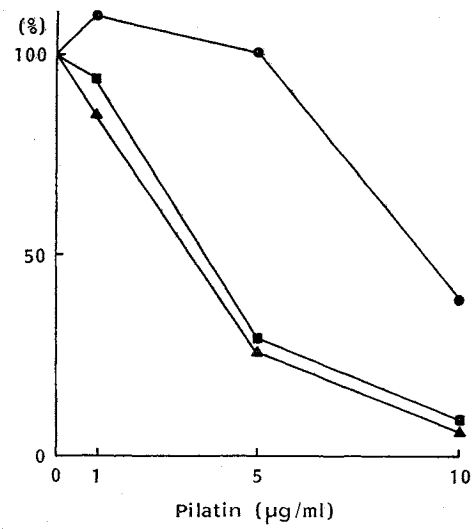
Table 3. Antifungal activity of pilatin (**1**) and marasmic acid (**3**) in the plate diffusion assay.

	Diameter of inhibition zones (mm)	
	Pilatin	Marasmic acid
Ascochyta pisi	—	—
Botrytis cinerea	15	10
Ceratocystis fimbriata	50	65
Mucor hiemalis	13	16
Phytophthora infestans	—	8
Pleospora herbarum	22	15
Sclerotinia fructigena	—	35

50 μg were applied to paper discs of 6 mm diameter.

Fig. 2. Effect of pilatin on the synthesis of macromolecules in Ehrlich carcinoma ascites cells in percent of the controls without antibiotic.

● Protein synthesis, ■ RNA synthesis, ▲ DNA synthesis.



Controls without antibiotics; incorporation per 3×10^6 cells: [^{14}C]Leucine, 38,937 cpm; [^{14}C]-uridine, 31,869 cpm; [^{14}C]thymidine, 2,772 cpm.

at room temperature the singlet of 5-H at δ 6.08 is significantly broadened.

The relative configuration of pilatin was determined by nuclear Overhauser effect (NOE) difference measurements. The results are given in Table 1 and indicate a *trans*-arrangement of the ester chain and the 9-hydroxy group, a *cis*-relationship between the 9-hydroxy group and the cyclopropyl ring and a *cis*-fusion of the rings.

On mild basic hydrolysis, **1** was converted into pilatol (**2**). The CD curves of **2** and marasmic acid **3** (Fig. 1) are in good agreement and prove that both compounds have the same absolute configuration[11]. The relative and absolute configuration of pilatin is given in formula **1**.

Pilatin exhibits both antibacterial and antifungal activities. In the serial dilution assay (Table 2) the MICs are somewhat lower as compared to marasmic acid. In the plate diffusion assay both compounds inhibited the growth of phytopathogenic fungi to a similar extent with the exception of *Sclerotinia fructigena*, which was sensitive only to marasmic acid (Table 3). Compared to pilatin and marasmic acid, the antimicrobial activity of pilatol (**2**) is markedly reduced. The MICs for *B. brevis*, *Bacillus subtilis*, and *Nematospora coryli* are 10 times higher. The cytotoxic effect of pilatin is quite

Table 4. Mutagenic activity of pilatin (1) and marasmic acid (3) in the assay of AMES *et al.*[6].

Compound	μg/disc	Number of revertants *Salmonella typhimurium* strain			
		TA 1535	TA 1538	TA 100	TA 98
MNNG[a]	5	2,944 (13)[b]	— (12)	20,750 (12)	— (11)
Pilatin	100	— (15)	— (22)	— (18)	148 (25)
Marasmic acid	50	— (32)	— (36)	— (35)	344 (50)

[a] *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine.
[b] Diameter of inhibition zones (mm).
—: No revertants above background.

high. In cells of the ascitic form of Ehrlich carcinoma the incorporation of [^{14}C]thymidine and [^{14}C]-uridine into trichloroacetic acid precipitable material (DNA, RNA) is strongly inhibited by 5 μg/ml of pilatin, whereas the incorporation of [^{14}C]leucine into protein is affected only at higher concentrations (Fig. 2). In a very similar assay[12] macromolecular syntheses of both normal and Rous sarcoma virus-transformed chicken embryo fibroblasts were inhibited to the same extent and by the same concentrations of pilatin (5~10 μg/ml). At these concentrations, pilatin did not interfere with the uptake of the precursors leucine, uridine, and thymidine. Very similar results have been obtained for marasmic acid[13]. The interference of pilatin with nucleic acid biosyntheses led us to test the mutagenic potential in the *S. typhimurium* assay of AMES *et al.*[6]. In the spot test 100 μg of pilatin/disc increased the number of revertants from *S. typhimurium* TA 98 indicating frameshift mutations (Table 4). The mutagenic activity, however, was lower as compared to marasmic acid and minute as compared to the control mutagen MNNG.

Acknowledgment

We thank the Deutsche Forschungsgemeinschaft for financial support. The technical assistance of Miss M. PRAEMASSING is gratefully acknowledged.

References

1) BACKENS, S.; W. STEGLICH, J. BÄUERLE & T. ANKE: Antibiotics from basidiomycetes. XXVIII. Hydroxystrobilurin D, ein antifungisches Antibiotikum aus Kulturen von *Mycena sanguinolenta* (*Agaricales*). Liebigs Ann. Chem. 1988: 405~409, 1988
2) AGERER, R.: *Flagelloscypha*. Studien an cyphelloiden basidiomyceten. Sydowia 27: 131~265, 1975
3) ANKE, T. & W. STEGLICH: Neue Wirkstoffe aus Basidiomyceten. Forum Mikrobiologie 11: 21~25, 1988
4) 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
5) KUPKA, J.; T. ANKE, F. OBERWINKLER, G. SCHRAMM & W. STEGLICH: Antibiotics from basidiomycetes. VII. Crinipellin, a new antibiotic from the basidiomycetous fungus *Crinipellis stipitaria* (FR.) PAT. J. Antibiotics 32: 130~135, 1979
6) AMES, B. N.; J. MCCANN & E. YAMASAKI: Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian microsome mutagenicity test. Mutat. Res. 31: 347~364, 1975
7) TURNER, D. L.: Carbon-13 autocorrelation NMR using double-quantum coherence. J. Magn. Reson. 49: 175~178, 1982
8) MARECI, T. H. & R. FREEMAN: Echoes and antiechoes in coherence transfer NMR: Determining the signs of double-quantum frequencies. J. Magn. Reson. 48: 158~163, 1982
9) BAX, A. & T. H. MARECI: Practical aspects of carbon-13 double quantum NMR. J. Magn. Reson. 53: 360~363, 1983
10) BAX, A. & G. A. MORRIS: An improved method for heteronuclear chemical shift correlation by two-dimensional NMR. J. Magn. Reson. 42: 501~505, 1981
11) CRADWICK, P. D. & G. A. SIM: Crystallographic determinations of partial stereochemistry of the ses-

quiterpenoids illudol and marasmic acid. Chemical Commun. 1971: 431~432, 1971

12) KUPKA, J.; T. ANKE, W. STEGLICH & L. ZECHLIN: Antibiotics from basidiomycetes. XI. The biological activity of siccayne, isolated from the marine fungus *Halocyphina villosa* J. & E. KOHLMEYER. J. Antibiotics 34: 298~304, 1981

13) 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