Phellodonic Acid, a New Biologically Active Hirsutane Derivative from *Phellodon melaleucus* (Thelephoraceae, Basidiomycetes) [1]

Marc Stadler, Timm Anke

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

Johannes Dasenbrock and Wolfgang Steglich

Institut für Organische Chemie, Ludwig-Maximilians-Universität, Karlstraße 23, D-80333 München, Bundesrepublik Deutschland

Dedicated to Dr. habil. Siegfried Huneck on the occasion of his 65th birthday

Z. Naturforsch. **48c,** 545–549 (1993); received April 23, 1993

Phellodon melaleucus, Sesquiterpenoids, Hirsutane, Phellodonic Acid, Antibiotic

A new hirsutane derivative, phellodonic acid (**1**), has been isolated from fermentations of *Phellodon melaleucus* strain 87113. Its structure was elucidated by spectroscopic methods. The compound exhibits antibiotic activities towards bacteria and fungi. **1** is the first bioactive metabolite from cultures of a species belonging to the family Thelephoraceae.

Introduction

The genus *Phellodon* belongs to the hydnaceous Basidiomycetes and is included within the family Thelephoraceae. Eight species have been described to occur in the Asiatic-Australian region by Maas Geesteranus [2]. Up to now no biologically active compounds have been described from the genus. In the following we wish to report the fermentation, isolation, structure elucidation and biological evaluation of a new antibiotic from *Phellodon melaleucus*.

Materials and Methods

Phellodon melaleucus strain TA 87113

Mycelial cultures of *Phellodon melaleucus* (Sw. ap. Fr. ex Fr.) P. Karst. strain TA 87113 were obtained from a spore print of a fruiting body collected at the Hartz Mountains, Tasmania, in 1987. Voucher specimen and mycelial cultures are deposited at the Lehrbereich Biotechnologie, Universität Kaiserslautern.

Reprint requests to Prof. Dr. T. Anke or Prof. Dr. W. Steglich.

Verlag der Zeitschrift für Naturforschung, D-72072 Tübingen
0939–5075/93/0700–0545 $ 01.30/0

Fermentation

For maintenance on agar slants and submerged cultivation, the strain was grown in YMG medium composed of (g/l): glucose 4, malt extract 10, yeast extract 4. The pH was adjusted to 5.5 before autoclaving. Fermentations were carried out in a Biolafitte C-6 apparatus containing 20 l of YMG medium with agitation (180 rpm) and aeration (3.2 l/min) at 24 °C. The inoculum was 400 ml of a well grown culture in the same medium. Antibacterial activity *(Bacillus brevis)* was determined by plate diffusion assay using 10 µl aliquots of concentrated (100-fold) ethyl acetate extracts of the culture fluid.

Isolation of phellodonic acid (**1**)

After nine days of fermentation the mycelia were separated by filtration and discarded. The culture filtrate (17 l) was then acidified to pH 4 and applied to a column (6 × 30 cm) containing Mitsubishi HP 21 resin and washed with 5 l of water. Phellodonic acid was eluted from the column with 3 l of acetone. After evaporation of the acetone the antibiotic was extracted from the residual aqueous phase with two times 750 ml of ethyl acetate. The crude product (1.8 g) obtained after removal of the solvent was purified by MPLC on silicic acid [Sigma SIL A 200, column 2.5 × 25 cm, elution with cyclohexane–EtOAc–EtOH (85:15:1)] yielding 360 mg of an enriched product. Final purification was achieved by HPLC

on LiChro Gel PS 1 (Merck, 10 μm) using 2-propanol as eluant. Yield: 37 mg of **1**.

Physico-chemical properties of phellodonic acid (**1**)

Colourless oil, soluble in methanol, acetone, chloroform, dichloromethane, cyclohexane and toluene, R_f 0.82 [toluene–acetone–EtOAc (70:30:1)], R_f 0.88 [toluene–HCO_2Et–HCO_2H (10:5:3)], $[\alpha]_D^{18}$ −114 (*c* 1.67, $CHCl_3$); UV (MeCN) λ_{max} 234 nm (log ε 3.80); CD (MeCN): $[\Theta]_{235}$ 11.32×10^3, $[\Theta]_{243}$ 0, $[\Theta]_{249}$ -5.66×10^3, $[\Theta]_{287}$ 0, $[\Theta]_{353}$ -16.63×10^3, $[\Theta]_{364}$ -15.81×10^3; IR (KBr) cm^{-1} 3500 (m), 2945 (st), 2930 (sst), 2870 (m), 2855 (m), 1740 (sh), 1735 (sst), 1640 (m), 1570 (m), 1465 (m), 1390 (m), 1295 (st), 1285 (st), 1250 (st), 1235 (st), 1170 (st), 1100 (st), 1070 (m), 1015 (m), 1020 (m), 945 (w), 930 (w), 735 (w), 700 (w); ^1H and ^{13}C NMR spectra see Table I; HR-MS (70 eV; DI 180 °C): *m/z* (relative intensity, %) 404.2225 (0.7, M^+, calcd for $C_{23}H_{32}O_6$ 404.2199), 262 (3.1, $C_{15}H_{18}O_4$), 261 (12.0, $C_{15}H_{17}O_4$), 260 (61.5, $C_{15}H_{16}O_4$), 245 (4.8, $C_{14}H_{13}O_4$), 232 (8.6, $C_{14}H_{16}O_3$), 231 (8.3, $C_{14}H_{15}O_3$), 216 (19.5, $C_{14}H_{16}O_2$), 215 (100, $C_{14}H_{15}O_2$), 214 (11.0, $C_{14}H_{14}O_2$), 204 (6.8, $C_{13}H_{16}O_2$), 203 (24.7, $C_{13}H_{15}O_2$), 127 (13.0, $C_8H_{16}O_2$), 57 (61.0); FAB$^+$-MS: *m/z* 427 ($M + Na^+$), 405 ($M + H^+$), 387 ($M^+ - OH$).

Biological assays

The assays for antimicrobial activity were performed as described previously [3]. *Chlorella vulgaris* was grown under the condition described by Noll [4]. The cytotoxicity of **1** against cells of the ascitic form of Ehrlich carcinoma (ECA, mouse), L 1210 (mouse), BHK 21 (hamster), and HeLa S3 cells (human) was measured as described previously [5]. The effect of **1** on the incorporation of [^{14}C]thymidine, [^{14}C]uridine, and [^{14}C]leucine into DNA, RNA, and proteins in L 1210 cells was measured as described previously for ECA cells [6]. The incorporations of radiolabelled precursors into the corresponding macromolecules were determined as described in [7].

Results and Discussion

Fermentation and isolation

A typical fermentation diagram is shown in Fig. 1. The appearance of antibiotic activity starts early in the growth phase of the producer. The

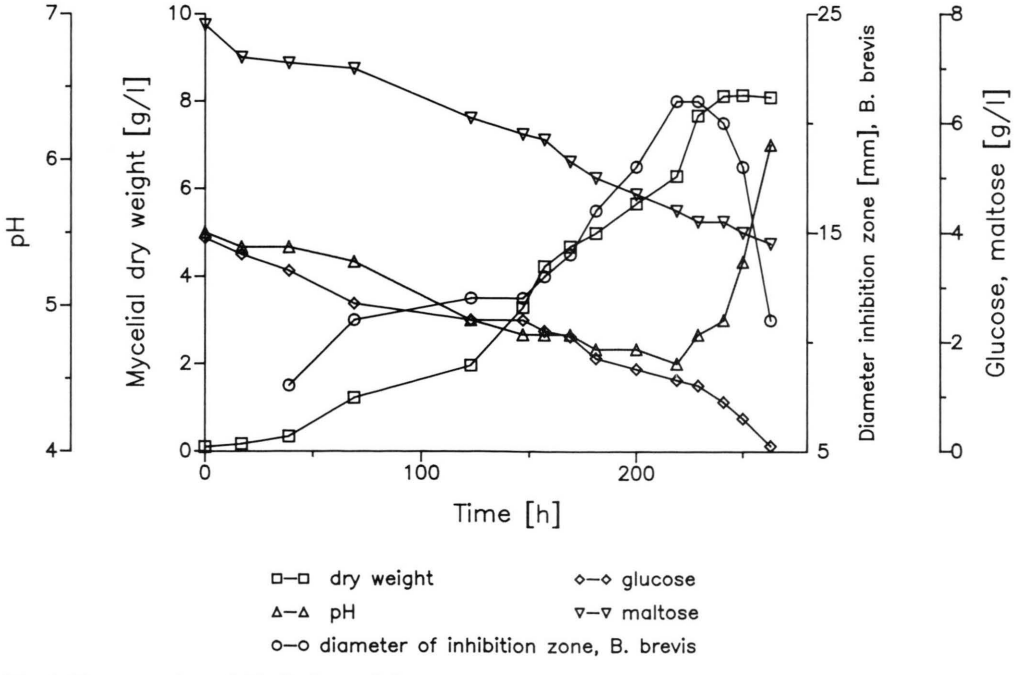


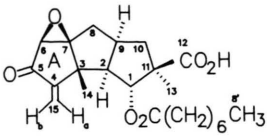
Fig. 1. Fermentation of *Phellodon melaleucus*.

highest antibiotic content in the culture was reached after 9 days. After all chromatographic steps 2.18 mg/l of pure phellodonic acid (**1**) was obtained.

Structure elucidation

Phellodonic acid (**1**) exhibits a UV maximum at 234 nm and IR absorptions at 1735 and 1640 cm^{-1} which indicate the presence of an α,β-unsaturated carbonyl system. The EI and FAB^+ mass spectra reveal the molecular ion $[M^+]$ at *m/z* 404 corresponding to the molecular formula $C_{23}H_{32}O_6$. The fragmentation pattern showed subsequent losses from the molecular ion of 144 (loss of octanoic acid) and of 44 (loss of CO_2) leading to the base peak at *m/z* 215. This indicates the presence of an octanoic acid moiety esterified with a sesquiterpenoid hydroxy acid $C_{15}H_{16}O_4$.

The ^1H and ^{13}C NMR spectra (Table I) confirm this conclusion and point to a close structural relationship between phellodonic acid and the hirsutane derivatives complicatic acid (**2**) [8, 9] and hypnophilin (**3**) [9]. Thus, a comparison with the ^{13}C NMR spectra of these antibiotics indicates that ring A is also present in phellodonic acid. This is corroborated by the characteristic long range coupling [^5J (H,H) < 1 Hz] of the epoxide proton at δ 3.43 with proton H_a of the *exo*-methylene group at δ 5.35 [9, 10]. An analysis of the remaining ^1H and ^{13}C NMR signals leads to structure **1** for phellodonic acid which is supported by $^1H-^1H$-COSY and COLOC experiments [11].



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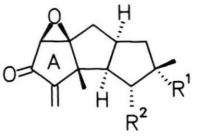


2, $R^1 = CO_2H$, $R^2 = H$
3, $R^1 = CH_3$, $R^2 = OH$

In the ^1H NMR the doublet at δ 5.80 can be attributed to the proton at C-1 carrying the octanoic acid residue. Its coupling constant $^3J = 7$ Hz is in accord with a *trans*-relationship to the angular proton in 2-position [9]. This is confirmed by the difference NOE spectrum which indicates a *cis*-relationship for 1-H and Me-14. Further NOEs, indicated in Fig. 2, allow the assessment of the relative stereochemistry for phellodonic acid. Thus, irradiation of the angular 9-H enhances the signals of 2-H, Me-13 and 6-H (weakly) which must therefore by positioned at the same side of the molecule. The carboxyl group at C-11 is therefore *trans*-

Table I. ^1H and ^{13}C NMR data of phellodonic acid (**1**) (400 and 100.6 MHz, respectively, δ-values; ^1H NMR in $CDCl_3$, ^{13}C NMR in C_6D_6 with TMS as internal standard).

H[a]	δ	Multiplicity	C[b]	δ	C	δ
1-H	5.80	d, $J = 7$ Hz	C-1	77.77	C-1′	172.68
2-H	2.19–2.32[c]	m	C-2	54.20	C-2′	34.26
			C-3	46.15	C-3′	25.27
			C-4	152.86	C-4′	29.29[d]
			C-5	196.77	C-5′	29.24[d]
6-H	3.43	s (br)	C-6	60.77	C-6′	31.98
			C-7	75.86	C-7′	22.96
8-H	1.90–2.18	m	C-8	30.16	C-8′	14.29
9-H	2.87	m	C-9	35.93		
10-H	1.90–2.18	m	C-10	43.67		
			C-11	54.13		
			C-12	181.52		
13-H	1.14–1.31[c]		C-13	16.78		
14-H	1.40	s	C-14	17.52		
15-H_a	5.35	s (br)	C-15	121.03		
15-H_b	6.05	s				

[a] 2′-H: δ 2.19–2.32 m[c]; 3′-H: δ 1.55, "pentet"; 4′-H, 5′-H, 6′-H, 7′-H: δ 1.14–1.31, m; 8′-H: δ 0.86 t, $J = 6.5$ Hz.
[b] Assignments confirmed by C,H-correlation experiments.
[c] Signals obscured.
[d] Signals may be interchanged.

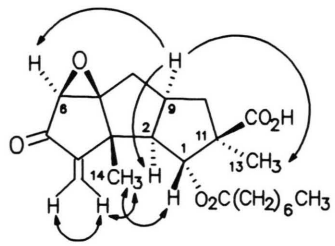
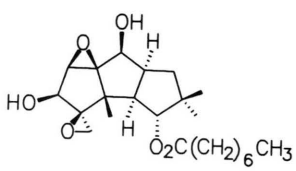


Fig. 2. NOEs observed in the ^1H NMR of phellodonic acid (**1**).

oriented with respect to the neighbouring octanoyloxy residue.

The absolute configuration of phellodonic acid given in the formula is assigned by the close agreement of its CD spectrum with that of complicatic acid (**2**) and hypnophilin (**3**) [9]. All three compounds exhibit a negative Cotton effect near 370 nm and a positive Cotton effect near 235 nm. It is interesting to note that a 1 α-octanoyloxy residue is also present in the hirsutane derivative coriolin B (**4**) [12]. Phellodonic acid differs from the known hirsutane carboxylic acids complicatic acid (**2**) and hirsutic acid C (**5**) [13] in its configuration at C-11.



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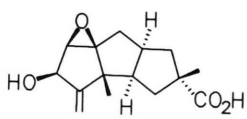

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Biological activities

The antimicrobial activities of phellodonic acid are summarized in Tables II and III. **1** exhibits rather strong inhibitory activities towards bacteria, yeasts, filamentous fungi and the alga *Chlorella vulgaris*. Interestingly, the producing strain is not sensitive to high concentrations of its own antibiotic (100 µg/disc in the plate diffusion assay). **1** is a highly cytotoxic compound (Table IV). At concentrations from 2–10 µg/ml lysis of all cell lines tested was observed. The incorporation of the radioactive precursors thymidine, uridine, and leucine into trichloroacetic acid-precipitable material (DNA, RNA, proteins) was reduced 50% by 1 µg/ml of **1** (Fig. 3). Phellodonic acid contains a highly reactive exomethylene group adjacent to a carbonyl function which readily reacts with thiols like cysteine forming an adduct which is completely devoid of antibiotic and cytotoxic activities. In sum-

Table II. Antimicrobial and algicidal activities of **1** in the serial dilution assay. The minimal inhibitory concentrations (MIC) were evaluated after 24 h incubation.

Organism	MIC [µg/ml]
Bacteria:	
Acinetobacter calcoaceticus	25
Bacillus brevis	2
Bacillus subtilis	5
Escherichia coli K 12	100
Micrococcus luteus	10
Staphylococcus aureus	10
Proteus vulgaris	10
Pseudomonas fluorescens	>100
Yeasts:	
Candida albicans	25
Nadsonia fulvescens	25
Nematospora coryli	10
Rhodotorula glutinis	100
Saccharomyces cerevisiae α S 288 c	25
Saccharomyces cerevisiae is 1	10
Algae:	
Chlorella vulgaris	25

Table III. Antifungal activities of **1** in the plate diffusion assay.

Organism	Diameter of inhibition zone [mm] 100 µg/paper disc (∅ 6 mm)
Mucor miehei	35
Paecilomyces variotii	32
Penicillium notatum	33
Ustilago nuda	37
Phellodon melaleucus	–

– = no inhibition zone.

Table IV. Cytotoxic effects of **1**.

Cell line	IC_{100}* [µg/ml]
BHK 21	5
ECA	5
L 1210	2
HeLa S 3	10

* 100% inhibition of growth.

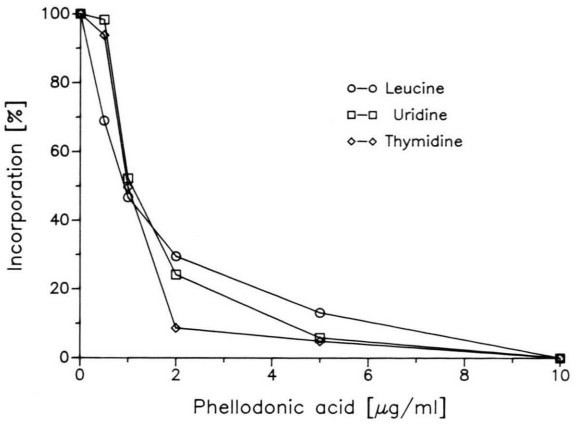

Fig. 3. Effects of **1** on the incorporation of labelled precursors into DNA, RNA, and proteins of L 1210 cells in percent of the controls without antibiotic. Controls, incorporation into TCA-precipitable material: [^{14}C]thymidine, 4123 cpm; [^{14}C]uridine, 7209 cpm; [^{14}C]leucine, 14121 cpm.

mary, the biological activities of **1** closely resemble those of the related hirsutane derivatives hypnophilin (**3**), pleurotellol, pleurotellic acid, and complicatic acid (**2**) [9].

Acknowledgements

The financial support of the Bundesminister für Forschung und Technologie is gratefully acknowledged. We thank Dr. Bert Steffan, München, for the NMR experiments and Dr. G. Eckhardt, Bonn, for the mass spectra.

[1] Antibiotics from Basidiomycetes, XLII. XLI: G. Erkel, T. Anke, A. Gimenez, and W. Steglich, J. Antibiot. **45,** 29–37 (1992).
[2] R. A. Maas Geesteranus, The Hydnaceous Fungi of the Eastern Old World, pp. 26–41, North Holland Publishing, Amsterdam 1971.
[3] J. Kupka, T. Anke, F. Oberwinkler, G. Schramm, and W. Steglich, J. Antibiot. **32,** 130–135 (1979).
[4] H. Noll, Doctoral Thesis, Kaiserslautern 1987.
[5] G. Erkel, T. Anke, R. Velten, and W. Steglich, Z. Naturforsch. **46c,** 442–450 (1991).
[6] W. Weber, T. Anke, B. Steffan, and W. Steglich, J. Antibiot. **43,** 207–212 (1990).
[7] T. Anke, F. Oberwinkler, W. Steglich, and G. Schramm, J. Antibiot. **30,** 806–810 (1977).
[8] G. Mellows, P. G. Mantle, T. C. Feline, and D. J. Williams, Phytochemistry **12,** 2717–2720 (1973).
[9] J. Kupka, T. Anke, B. M. Giannetti, and W. Steglich, Arch. Microbiol. **130,** 223–227 (1981).
[10] T. Anke, J. Heim, F. Knoch, U. Mocek, B. Steffan, and W. Steglich, Angew. Chem. **97,** 714–716 (1985); Angew. Chem., Int. Ed. Engl. **24,** 711–712 (1985).
[11] H. Kessler, C. Griesinger, J. Zarbock, and H. R. Loosli, J. Magn. Reson. **57,** 331–336 (1984).
[12] S. Takahashi, H. Naganawa, H. Iinuma, T. Takida, K. Maeda, and H. Umezawa, Tetrahedron Lett. **1971,** 1955–1958.
[13] F. W. Comer, F. McCapra, I. H. Qureshi, and A. I. Scott, Tetrahedron **23,** 4761–4768 (1967).