(—)-MITORUBRINOL AND PHTHALDEHYDIC ACIDS, NEW METABOLITES OF *Penicillium vermiculatum* DANG.

Bohumil PROKSA[a], Dušan UHRÍN[a], Ján FUSKA[b] and Eva MICHÁLKOVÁ[c]

[a] *Institute of Chemistry, Slovak Academy of Sciences, 842 38 Bratislava*
[b] *Department of Biochemical Technology, Slovak Technical University, 812 37 Bratislava*
[c] *Drug Research Institute, 976 13 Slovenská Ľupča*

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Three new metabolites — (—)-mitorubrinol, 2-formyl-3,5-dihydroxy-4-methylbenzoic and 2-formyl-3,5-dihydroxy-4-hydroxymethylbenzoic acids — were isolated from the culture medium of *Penicillium vermiculatum* DANG. Hexane extract of the mycelium contained ergosterol and esters of fatty acids, linoleic, oleic and palmitic acids being the dominating ones.

Main metabolites of *Penicillium vermiculatum* DANG. are — according to the composition of the culture medium — the macrolide dilactone (—)-vermiculin[1] or the phthalide derivative (—)-vermistatin[2]. In addition to these substances further minor components were produced; this paper concerns their isolation and identification.

Vermiculin was separated from the chloroform extract of the *P. vermiculatum* mycelium by crystallization from methanol. The mother liquor was extracted with hexane to remove the fatty compounds and chromatographed to yield the pigment *I* ($C_{21}H_{18}O_8$). Its ultraviolet spectrum showing bands at 216, 266, 292 and 346 nm, which were bathochromically shifted in alkaline medium, is indicative of a conjugated system of double bonds in the neighbourhood of an ionizable group of the compound under investigation. The IR spectrum revealed bands of an ester group at 1 715 cm^{-1} and those of an α,β-unsaturated ketone (1 660 cm^{-1}). A successive disappearance of the proton signal (δ 5·67) associated with an sp^2 carbon was observed in the ^1H NMR spectrum (Table I). The presumed exchange of this proton for deuterium was corroborated by a regenerative exchange in a non-deuterated methanol. An unambiguous information on the structure of compound *I* provided the long-range coupling constants identified by an INEPT experiment[3] showing that the molecule of compound *I* composed of two relatively individual moieties coupled by an ester bond is identical with (—)-mitorubrinol isolated already from *P. rubrum*[4-6]. Conformation of ring B of compound *I* was determined from the NOE experiment: irradiation of the C-9 methyl group proton was manifested by

an enhanced intensity of the H-1 proton (8%); this indicated an axial arrangement of the methyl group bonded to the 7,8-dihydroxy-6*H*-2-benzopyrane ring system. The absolute configuration of compound *I* was derived from comparison of its CD spectrum with those of austdiol, (−)- and (+)-sclerotiorins[5]. Since the CD spectrum of (−)-sclerotiorin followed the same course, the configuration of (−)-mitorubrinol (*I*) at C-7 is *R*. (−)-Mitorubrinol belongs to the azaphilone group showing the so called averse effect[7] causing changes in morphology of various moulds[8].

The filtrate after removal of the *P. vermiculatum* mycelium was extracted with ethyl acetate–2-propanol, the extract was distributed between aqueous $NaHCO_3$ and the solvent to afford the mixture of acids, which was chromatographed to yield the components *II* ($C_9H_8O_5$) and *III* ($C_9H_8O_6$). The ^1H NMR spectrum of acid *II* (Table II) revealed signals of a methyl group at an sp^2 carbon, one proton of a substituted benzene ring and one proton of an aldehyde group. The ^1H NMR spectrum of compound *III* disclosed a signal of a CH_2OH group instead of that of the methyl group. The ^{13}C NMR spectra of compounds *II* and *III* are indicative of a substituted

TABLE I

The NMR data of pigment *I* (δ, ppm; CD_3OD)

Position	^{13}C	^1H
1	155·3	8·10 d ($J_{1,5}$ = 1·2 Hz)
3	156·9	—
4	110·4	6·44 s
4a	145·0	—
5	107·6	5·67 d ($J_{5,1}$ = 1·2 Hz)
6	194·0	—
7	85·7	—
8	193·5	—
8a	115·5	—
9	22·7	1·68 s
1′	120·1	6·39 dt ($J_{1',2'}$ = 15·6 Hz; $J_{1',3'}$ = 1·6 Hz)
2′	139·8	6·72 dt ($J_{2',1'}$ = 15·6 Hz; $J_{2',3'}$ = 4·2 Hz)
3′	61·9	4·32 dd ($J_{3',1'}$ = 1·6 Hz; $J_{3',2'}$ = 4·2 Hz)
1″	104·3	—
2″	165·8	—
3″	101·3	6·20 dq ($J_{3'',5''}$ = 2·5 Hz; $J_{3'',7''}$ = 0·5 Hz)
4″	163·8	—
5″	112·5	6·27 dq ($J_{5'',3''}$ = 2·5 Hz; $J_{5'',7''}$ = 0·8 Hz)
6″	144·9	—
7″	24·2	2·65 dd ($J_{7'',3''}$ = 0·5 Hz; $J_{7'',5''}$ = 0·8 Hz)
8″	170·6	—

phthalaldehydic acid. Arrangement of substituents at this ring system was determined by a heterocorrelated INEPT experiment. A magnetization transfer from the methyl group proton of compound *II* was observed with signals at δ 164·8, 163·1 and 116·3; this finding together with the low value for the shift of the methyl group corresponded with the structure of 2-formyl-3,5-dihydroxy-4-methylbenzoic acid. This assignment was corroborated by the interaction of both the proton resonating at δ 7·04 with carbons at δ 169·1, 116·1 and 113·0, and the aldehyde group proton with carbons resonating at δ 164·8 and 113·0. The 2-formyl-3,5-dihydroxy-4-methylbenzoic acid has already been isolated from the culture medium *P. funiculosum* THOM. under the trivial name funiculosic acid[9]. The structure of compound *III* solved in an analogous way was shown to be 2-formyl-3,5-dihydroxy-4-hydroxymethylbenzoic acid; this substance, which is a degradation product of funiculosic acid has not been described as yet. The unsubstituted 2-formylbenzoic acid (phthalaldehydic acid) cyclizes easily to furnish 3-hydroxyphthalide; the equilibrium in neutral and acid solutions is unequivocally shifted in favour of the phthalide[10]. The isolated acids *II* and *III* dissolved in methanol were relatively stable; formation of phthalides *IV* and *V* was observed after some days only. Decrease of acids *II* and *III* was seen in the NMR spectra as a disappearance of the aldehyde and H-6 proton signals concurrently with the increase of phthalide H-3 and H-7 proton intensities. Comparison of ^{13}C NMR shifts of compounds *IV* and *V* (Table III) with those reported[11] for phthalides *VI* and *VII* confirmed the proposed structure of compounds *II* – *V*. Of significance in this comparison were mainly shifts of carbons C-3a and C-7a. Acid *II* was chemically characterized by preparation of its derivatives; thus, treatment of *II* with acetic

TABLE II
Chemical shifts of acids *II* and *III* (δ, ppm; CD_3OD)

Position	^{13}C		^{1}H	
	II	*III*	*II*	*III*
1	134·6	136·5	—	—
2	113·0	112·5	—	—
3	164·8	164·5	—	—
4	116·2	117·2	—	—
5	163·1	163·8	—	—
6	111·9	112·3	7·04 s	7·02 s
COOH	169·1	168·6		
CHO	197·0	196·6	10·46 s	10·44 s
CH_3	7·8	—	2·11 s	—
CH_2OH	—	54·3	—	4·80 s

anhydride in pyridine and hydrazine gave 3,4,6-triacetoxy-5-methylphthalide (*VIII*), and phthalazin-1-one (*IX*), respectively. The latter was acetylated into the diacetate *X*.

Acids *II* and *III* can be considered by-products in the biosynthesis of (−)-vermistatin (*XI*), the structure of which embodies the unusual 4,6-dimethoxyphthalic grouping[12]. It is, therefore, our belief that formation of compounds *II*, *III* and *XI* is associated with the orselinic acid metabolism; isolation of (−)-mitorubrinol in which this acid is bonded in form of an ester also supported this hypothesis.

The hexane extract after separation of vermiculin was chromatographed to afford ester and sterol fractions. The ester fraction was hydrolyzed into fatty acids with C_{18}-acids dominating (Table IV), whilst ergosterol was found to prevail in the sterol portion.

TABLE III

^{13}C NMR shifts of phthalides *IV*— *VII* (δ, ppm; CD_3OD)

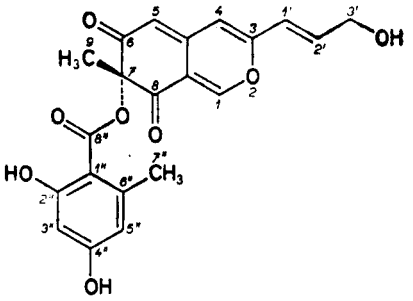
Position	*IV*	*V*	*VI*[a]	*VII*[a]
1	171·5	170·8	171·6	173·1
3	102·6	102·7	68·4	69·1
3a	123·2	122·9	125·2	49·6
4	152·7	153·7	150·7	104·2
5	120·7	120·2	120·3	163·5
6	160·4	159·8	160·9	118·9
7	103·7	103·5	98·4	159·2
7a	126·4	128·3	127·6	109·5
CH_3	9·2	—	9·2	8·7
CH_2OH	—	57·9	—	—
OCH_3	—	—	56·4	62·1

[a] CD_3COCD_3, ref.[11].

TABLE IV

Composition of the mixture of fatty acids isolated from *P. vermiculatum*

Acid	%	Acid	%
Myristic	0·3	Oleic	25·8
Palmitic	14·9	Linoleic	46·3
Palmitoleic	0·5	Linolenic	5·8
Stearic	5·9		



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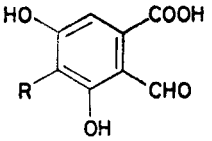
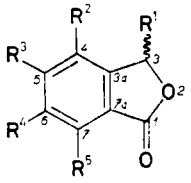
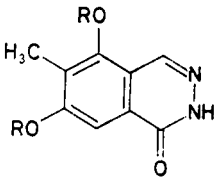


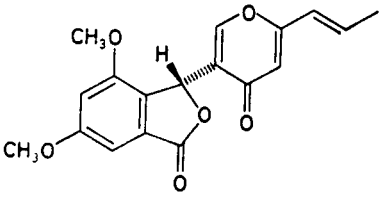
II, R = CH_3
III, R = CH_2OH



IV, R^1= R^2= R^4= OH ; R^3= CH_3 ; R^5= H
V, R^1= R^2= R^4= OH ; R^3= CH_2OH ; R^5= H
VI, R^1= R^5= H ; R^2= OH ; R^3= CH_3 ; R^4= OCH_3
VII, R^1= R^2= H ; R^3= OH ; R^4= CH_3 ; R^5= OCH_3
VIII, R^1= R^2= R^4= $OCOCH_3$; R^3= CH_3 ; R^5= H



IX, R = H
X, R = CH_3CO



XI

EXPERIMENTAL

Strain *Penicillium vermiculatum* DANG. CCM F-748 was used for cultivation. Composition of the substrate and cultivating conditions were the same as in production of vermistatin[1].

Melting points were determined on a Kofler micro hot-stage, the UV (λ in nm) and IR ($\tilde{\nu}$ in cm^{-1}) spectra were recorded with Specord UV VIS (Zeiss, Jena) and Perkin–Elmer model 983

spectrophotometers, respectively. The electron impact mass spectra were run with a Jeol JMS 100D apparatus at 70 eV ionization energy and 300 μA trap current. The ^1H and ^{13}C NMR spectra were measured with Bruker AM 300 instrument at 300 and 75 MHz, respectively. The CD spectra were recorded with a Dichrograph Mark III-S (Jobin Yvon), optical rotation with a Perkin–Elmer, model 241 polarimeter. The methyl esters of fatty acids were determined by gas chromatography on a Hewlett–Packard, model 5880A apparatus provided with a capillary column 50 × 0·32 mm coated with a 0·4 μm film of OV-101; column temperature: 50—80°C, temperature gradient: 5°C min^{-1}. Silufol UV 254 sheets were used for thin-layer chromatography in chloroform–methanol (9 : 1, S_1), chloroform–methanol–acetic acid (9 : 1 : 0·4, S_2), and chloroform–heptane (3 : 1, S_3).

Isolation of (—)-Mitorubrinol

The mycelium of *P. vermiculatum* DANG. removed from the culture medium (2·5 l) was extracted with chloroform (4 × 100 ml), the combined extracts were concentrated and the precipitated vermiculin was filtered off after addition of methanol (25 ml). The filtrate was extracted with hexane (2 × 20 ml), the methanol-containing layer was concentrated and the residue was chromatographed on a silica gel-packed column, chloroform–methanol (4 : 1) being the eluent. The fractions were monitored by thin-layer chromatography in S_1. Crystallization of the fraction showing a spot of R_F 0·20 from methanol–ether afforded *I* (28 mg), m.p. 230°C (decomp.), $[\alpha]_D^{20}$ −382° (*c* 1, dioxane). For $C_{21}H_{18}O_8$ (398·4) calculated: 63·32% C, 4·55% H; found: 63·25% C, 4·62% H. UV spectrum (MeOH): 216 (log ε 4·26), 266 (4·24), 293 (4·00), 346 (4·22); (0·1 mol l^{-1} KOH in MeOH): 246 (log ε 4·32), 320 (4·30), 349 (4·47), 490 (3·67). IR spectrum (KBr): 3 400 (O—H), 1 715, 1 660 (C=O), 1 604, 1 550. CD spectrum: $\Delta\varepsilon_{355}$ −11·0, $\Delta\varepsilon_{308}$ +11·5, $\Delta\varepsilon_{245}$ −5·06. NMR spectrum see Table I.

Isolation of Acids *II* and *III*

The filtrate after separation of the mycelium (2 l) was extracted with ethyl acetate–2-propanol (4 : 1, 3 × 250 ml), the combined organic layers were concentrated, the residue was dissolved in ethyl acetate (50 ml) and extracted with aqueous $NaHCO_3$ (2%, 4 × 20 ml). The aqueous phase was adjusted to pH 2·0 and extracted with ethyl acetate (5 × 15 ml), the solvent was concentrated and the residue was chromatographed on a silica gel-packed column by a gradient elution with chloroform–methanol. The individual fractions were monitored by thin-layer chromatography in S_2. The combined fractions revealing R_F 0·44 were crystallized from ether–methanol to yield the acid *II* (40 mg), m.p. 265°C (decomp.). For $C_9H_8O_5$ (196·2) calculated: 55·11% C, 4·11% H; found: 55·06% C, 4·16% H. IR spectrum (KBr): 3 397 (O—H), 2 974 (C—H); 1 695 (C=O), 1 592, 1 477. Mass spectrum, *m/z* (%): 196, M^+ (100), 178 (15), 168 (18), 150 (63), 123 (18). The NMR spectral data are listed in Table II.

3,4,6-Triacetoxy-5-methylphthalide (*VIII*) was obtained from the acid *II* on reaction with acetic anhydride in pyridine, work-out and crystallization from methanol; m.p. 197—199°C. For $C_{15}H_{14}O_8$ (322·3) calculated: 55·90% C, 4·38% H; found: 55·83% C, 4·30% H. IR spectrum ($CHCl_3$): 3 014 (C—H), 1 780 (C=O), 1 618, 1 581, 1 427. ^1H NMR spectrum ($CDCl_3$): 7·54 s, 1 H (H-7); 7·41 s, 1 H (H-3); 2·38, 2·33, 2·16 3 s, 9 H (3 × CH_3CO); 2·12 s, 3 H (C-5-CH_3).

Combined fractions of R_F 0·24 were concentrated and crystallized from toluene–methanol to furnish acid *III* (52 mg), m.p. 260°C (decomp.). For $C_9H_8O_6$ (212·2) calculated: 50·95% C, 3·80% H; found: 50·85% C, 3·77% H. IR spectrum (KBr): 3 326 (O—H); 2 904 (C—H); 1 695 (C=O), 1 627, 1 604, 1 572, 1 515, 1 486. Mass spectrum, *m/z* (%): 212, M^+ (46), 194 (100), 176 (24), 166 (17), 148 (39). The NMR spectral data are presented in Table II.

5,7-Dihydroxy-6-methyl-1,2-dihydrophthalazin-1-one (*IX*)

Acid *II* (300 mg) dissolved in methanol (25 ml) was heated with hydrazine (80%, 1 ml) for 2 h. The solution was concentrated, the residue was dissolved in water, the pH was adjusted to 4 and the separated precipitate was filtered off and crystallized from methanol–water (2 : 1) to give compound *IX* (280 mg), m.p. 260°C (decomp.). For $C_9H_8N_2O_3$ (192·2) calculated: 56·25% C, 4·20% H, 14·57% N; found: 56·18% C, 4·17% H, 14·46% N. IR spectrum (KBr): 3 307 (O—H, N—H), 3 142 (C—H), 1 624 (C=O, C=N). ^{1}H NMR spectrum (CD_3OD): 8·45 s, 1 H (H-4); 7·21 s, 1 H (H-8); 2·24 s, 3 H (C-6-CH_3).

5,7-Diacetoxy-6-methyl-1,2-dihydrophthalazin-1-one (*X*) was prepared from compound *IX* by treatment with acetic anhydride in pyridine; m.p. 255—256°C (methanol). For $C_{13}H_{12}N_2O_5$ (276·2) calculated: 56·57% C, 4·38% H, 10·14% N; found: 56·49% C, 4·30% H, 10·07% N. IR spectrum (KBr): 3 307 (N—H), 3 172 (C—H), 1 766 (COO), 1 657 (CON). ^{1}H NMR spectrum (CD_3OD): 8·28 s, 1 H (H-4); 7·96 s, 1 H (H-8); 2·50 s, 3 H, 2·40 s, 3 H (2 × CH_3CO); 2·18 s, 3 H (C-6-CH_3).

Isolation of Fatty Acids and Ergosterol

The hexane extract of the filtrate after removal of vermiculin was concentrated and chromatographed on an alumina-packed column with heptane–ethyl acetate (2 : 1), the content of fractions being monitored by thin-layer chromatography in S_3. Fraction of R_F 0·85 contained esters of fatty acids (105 mg); these were hydrolyzed with methanolic KOH (0·5 mol l^{-1}, 5 ml). The hydrolysate was concentrated, pH of the residue dissolved in water was adjusted to 2, the liberated acids were extracted with ether and the mixture of acids obtained was esterified with diazomethane. The methyl esters were analyzed by gas chromatography (Table IV). The fraction of R_F 0·30 was worked out and crystallized from ether–methanol to afford a compound (18 mg) identical with ergosterol according to m.p., IR, mass and NMR spectral data.

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