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Identification and Determination of Organic Acids in Cultivation Medium of *Penicillium vermiculatum* Dang

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Summary. [2*E*,7*E*]-4,9-dioxo-2,7-decadienoic, [2*E*,7*E*]-9-oxo-2,7-decadienoic and [2*Z*,4*E*]-2-methyl-2,4-hexadienoic acids were isolated from the filtrate of *Penicillium vermiculatum* Dang. The presence of [2*E*,2′*E*,7*S*,7′*E*]-4,9-dioxo-7-(4′,9′-dioxo-2′,7′-decadienoyloxy)-2-decenoic acid was confirmed by chromatography. HPLC was used for the determination of these acids in the cultivation medium.

Keywords. *Penicillium vermiculatum*; vermiculin; decadienoic acids; 2-methylsorbic acid; HPLC.

Identifizierung und Bestimmung von organischen Säuren im Kultivierungsmedium von *Penicillium vermiculatum* Dang

Zusammenfassung. [2*E*,7*E*]-4,9-dioxo-2,7-decadiensäure, [2*E*,7*E*]-9-oxo-2,7-decadiensäure und [2*Z*,4*E*]-2-methyl-2,4-hexa-diensäure wurden aus dem Kultivierungsmedium von *Penicillium vermiculatum* Dang isoliert. Die Anwesenheit von [2*E*,2′*E*,7*S*,7′*E*]-4,9-dioxo-7-(4′,9′-dioxo-2′,7′-decadienoyloxy)-2-decensäure wurde chromatographisch bestätigt. Im Filtrat von *P. vermiculatum* wurden diese Säuren mittels HPLC analysiert.

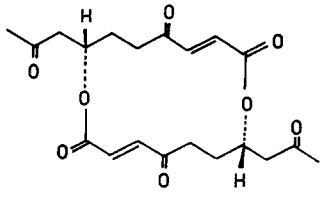
Introduction

The macrodiolide antibiotic vermiculin (**1**), a secondary metabolite of *Penicillium vermiculatum* Dang [1], showing remarkable *in vivo* immunoregulatory properties [2], is formally composed of two C_{10} aliphatic acids. During the vermiculin biosynthesis the *pH* of cultivation broth rapidly decreases to the end value around 3.0, which indicates the presence of organic acids. So far we have isolated *cis*-2-methylsorbic [3] and phthalaldehydic acids [4] from the filtrate of *P. vermiculatum*, but these acids have no apparent connectivity with the production of the main metabolite. The aim of this work was to identify proposed acidic precursors of vermiculin (**1**).

Results and Discussion

The residue after vermiculin crystallization distributed between chloroform and aqueous sodium bicarbonate solution afforded the mixture of organic acids, which

by chromatography on silica gel gave compounds **2**–**4**. Compound **2**, according to its MS, ^{1}H and ^{13}C NMR spectra, was identical with [2*E*,7*E*]-4,9-dioxo-2,7-decadienoic acid, which was recently identified in a mixture of compounds after vermiculin (**1**) degradation in boiling acetic acid [5].



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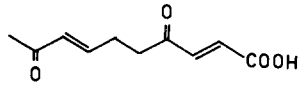

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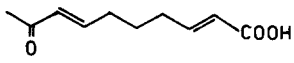

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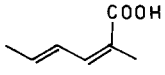

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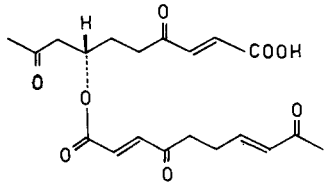

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Acid **3** afforded the MS peak of the parent ion at $m/z = 182$ ($C_{10}H_{14}O_3$). Two pairs of signals of protons bound to sp^2 carbons together with unresolved signals of four methylene protons 2.29 ppm) overlapping with a signal of one methyl group and that of one methylene group (1.68 ppm) were observed in the ^{1}H NMR spectrum. While the first pair of signals ($\delta = 6.11$ and 6.80 ppm) had similar shift values and the same splitting pattern as the protons H-7 and H-8 in the spectrum of compound **2**, the other pair ascribed to protons H-2 and H-3 was shifted upfield (5.86 and 7.05 ppm in **3** *vs.* 6.72 and 7.15 ppm in spectrum of compound **2**). Proton H-3 in structure **3** is adjacent to a methylene group and not to a carbonyl as was confirmed by the multiplicity of its signal (Table 1).

The ^{13}C-NMR spectrum of compound **3** displayed only 9 resolved signals, which were assigned to one ketone, one carboxyl, four protonated sp^2, two methylene and

Table 1. ^{1}H NMR data of isolated acids (ppm; δ relative to TMS; $CDCl_3$)

H	2	3
1	10.42 s	
2	6.72 d, 15.9	5.86 d, 15.9
3	7.15 d, 15.9	7.05 ddd, 15.9, 6.6, 6.6
4	–	2.29 m
5	2.88 m	1.68 m
6	2.60 m	2.29 m
7	6.83 ddd, 16.0, 6.6, 6.6	6.80 ddd, 16.0, 6.6, 6.6
8	6.13 dd, 16.0, 1.6	6.11 d, 16.0
10	2.26 d, 1.6	2.26 s

one methyl carbon, respectively (Table 2). According to the relative intensities, results of COSY and heterocorrelated experiments, two methylene groups had the same shift values in ^{13}C and ^{1}H-NMR (31.7 and 2.29 ppm, respectively). Supported by these results, compound **3** was ascribed the structure of [2*E*,7*E*]-9-oxo-2,7-decadienoic acid. Acid **4** was identical with [2*Z*,4*E*]-2-methyl-2,4-hexadienoic acid (**4**) [3].

Isolated compounds were sufficiently separated by HPLC on a RP-18 column (Fig. 1); the second peak in this chromatogram was ascribed to [2*E*,2′*E*,7*S*,7′*E*]-4,9-dioxo-7-(4′,9′-dioxo-2′,7′-decadienoyloxy)-2-decenoic acid (**5**) according to its retention time as well as its absorption bands in the UV spectrum. This assignment was confirmed also by comparison of the R_f value with that of the authentic sample and the color reactions with KOH/MeOH or vanilline/sulfuric acid. Due to different UV maxima, compounds **2**, **3** and **5** were monitored at 230 nm, while acid **4** was determined at 265 nm (Fig. 2). The mixture of organic acids isolated from the *P. vermiculatum* filtrate consisted of compounds **2** (36.61 ± 1.54%), **3** (7.12 ± 0.21%), **4** (22.16 ± 1.02%), and **5** (28.43 ± 1.17%). The cultivation medium of *P. vermiculatum*

Table 2. ^{13}C NMR data of isolated acids (ppm; δ relative to TMS; $CDCl_3$)

C	2	3
1	169.5	171.2
2	130.4	121.5
3	140.3	150.5
4	197.5	31.7
5	39.5	26.8
6	26.9	31.7
7	145.9	147.3
8	131.8	131.7
9	199.0	198.9
10	26.8	27.0

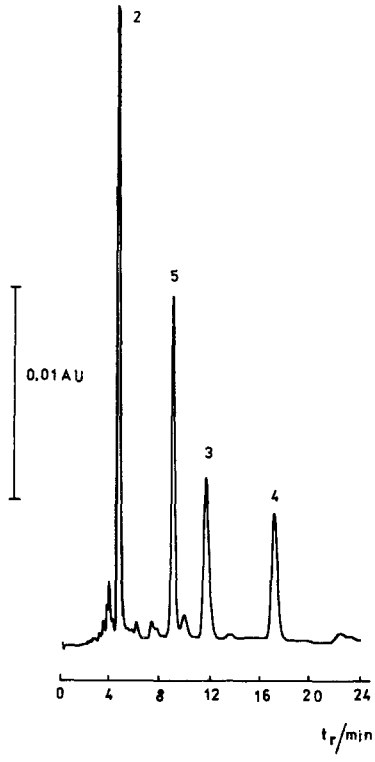

Fig. 1. Chromatogram of *P. vermiculatum* acids monitored at 230 nm. Conditions: column, Separon SGX C18 (7 μm) (150 × 3 mm); eluent, methanol:water (48:52), *pH* adjusted to 3.0 with H_3PO_4; flow rate 0.4 ml/min. For peak numbers see formulae

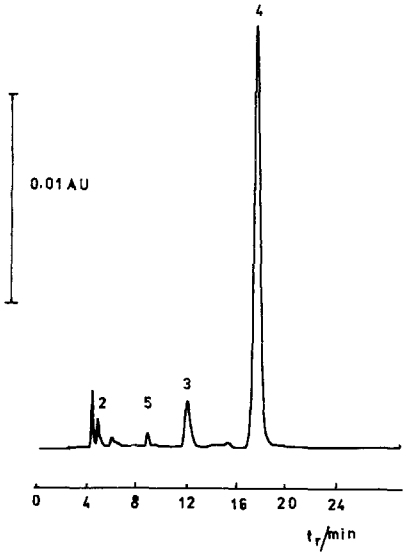

Fig. 2. Chromatogram of *P. vermiculatum* acids monitored at 265 nm. For conditions and peak numbers see Fig. 1

harvested in 144 h contained vermiculin (**1**) $[(2.10 \pm 0.16) \cdot 10^{-2}\%]$, acid 2 $[(8.10 \pm 1.01) \cdot 10^{-4}\%]$, acid **3** $[(0.22 \pm 0.03) \cdot 10^{-4}\%]$, 2-methylsorbic acid (**4**) $[(3.22 \pm 0.48) \cdot 10^{-4}\%]$, and acid **5** $[(9.00 \pm 0.94) \cdot 10^{-4}\%]$.

Experimental Part

Melting points: Kofler micro hot-stage; UV: Specord 40M (Zeiss, Jena); EI-MS: Jeol JMS 100D, ionization energy 70 eV, trap current 300 μA; ^1H and ^{13}C NMR: Varian VXR-300 at 300 and 75 MHz,

respectively; HPLC equipment (Laboratory Instruments, Prague): HPP 5001 pump, LCI 30 injector, LCD 2040 UV detector and CI-105 integrator; TLC: Silufol UV-254 (Kavalier, Votice, CR), chloroform-methanol-acetic acid = 90:10:1, visualisation at 254 nm or by spraying with vanillin/sulfuric acid or methanolic KOH.

Isolation of acids **2, 3,** *and* **4**

The liquors resulting from the crystallization of vermiculin from chloroform were concentrated, the residue (2.5 g) was dissolved in ethanol, the rest of vermiculin (450 mg) was filtered off, the filtrate was dried in vacuo, the residue was taken up in chloroform (50 ml) and extracted with aqueous sodium bicarbonate solution (twice with 20 ml, 3%). The combined aqueous extracts were acidified to *pH* 2.0 with diluted hydrochloric acid and extracted with chloroform 2-propanol (3:1). The organic solvents were removed in vacuo and the residue was chromatographed on silica gel column (chloroform: methanol = 9:1). Combination of appropriate eluates according to TLC with subsequent concentration and crystallization from hexane-acetone afforded [2*E*,7*E*]-4,9-dioxo-2,7-decadienoic acid (**2**, 247 mg) [5], [2*Z*,4*E*]-2-methyl-2,4-hexadienoic acid (**4**, 85 mg) [3], and [2*E*,7*E*]-9-oxo-2,7-decadienoic acid (**3**, 62 mg). M.p.: 69–72 °C; calc. for $C_{10}H_{14}O_3$ (182.2): 65.91% C, 7.74% H; found: 65.86% C, 7.81% H. NMR: Tables 1 and 2.

Determination of acids **2–5** *in cultivation medium*

Cultivation medium (5.0 g) was mixed with chloroform (2.0 ml, 5 min), the suspension was centrifuged (10 000 rpm, 3 min) and 12.0 ml of the chloroform layer were evaporated. The residue was dissolved in acetonitrile (0.5 ml) and 3 µl of this solution were injected onto the chromatographic column. Column: 150 × 3 mm, packed with Separon SGX C18 7 µm (Tessek, Prague); mobile phase: methanol: water = 42:58 (*pH* 3 adjusted with H_3PO_4); flow rate: 0.4 ml/min; wavelength of the UV detector: 230 nm for vermiculin (**1**) and acids **2**, **3** and **5**, 265 nm for 2-methylsorbic acid (**4**). Standards of all determined compounds were isolated and characterized in our laboratory. A linear relationship between peak area and concentration of determined compounds in the range 20–300 µg/ml was observed with regression coefficient r better than 0.97 for all compounds. Results were calculated for $n = 5$, $a = 0.05$.

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