Hydroxyfuniculosic and Funiculosic Acids, Metabolites of *Penicillium vermiculatum*

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Hydroxyfuniculosic and funiculosic acids were isolated as the major metabolites of *Penicillium vermiculatum* grown on saccharose medium. These acids together with vermistatin and (-)-mitorubrinic acid were identified in medium with glucose. Mechanism of biosynthesis of phthalaldehydic acids is discussed.

Biosynthesis of the secondary metabolites in microorganisms can be affected by external factors (*e.g.* composition of cultivation medium) or by changes in the genetic equipment of the cell (mutation, genetic recombination, *etc.*). Both these approaches were applied in preparation of new strains of *Penicillium vermiculatum*; here we present the results of this study.

P. vermiculatum DANG. CCM F-276 produced macrodiolide vermiculin (*I*) on saccharose medium [1], or phthalido-pyranone vermistatin (*II*) in the presence of glucose [2]. Three new stable mutant strains (designated PV-1, PV-2, and PV-3) of this parent strain were prepared by the active selection after UV irradiation combined with application of *N*-methyl-*N*-nitrosourea [3]. PV-1 and PV-2 differed only in their morphology, but only slightly in the spectrum of the biosynthesized metabolites. Substantial difference between these two strains and the parent one was in the ability to produce vermiculin (*I*) that mutant strains biosynthesized in higher amount, and, surprisingly, even in medium where saccharose was replaced by galactose.

Strain PV-3 did not form vermiculin (*I*) in saccharose medium or in medium completed with corn steep liquor which was prerequisite for biosynthesis of diolide *I*. Instead of this metabolite, two compounds giving the positive reaction with $FeCl_3$ and methyl orange solution were identified in both types of medium after cultivation of PV-3; these compounds according to spectral data were identical with hydroxyfuniculosic (*III*) and funiculosic (*IV*) acid [4]. Acids *III* and *IV* belong into phthalaldehydic acid group, in acidic media occurring in the form of hydroxyphthalides *V* and *VI*. Both acids as well as their transformation products were monitored by TLC and HPLC (Table 1). Acids *III* and *IV* appeared in medium after 96 h of cultivation when acid *IV* prevailed, but afterwards its concentration decreased in favour of the hydroxy analogue *III* (Table 2). Addition of acid *IV* into suspension of resting cells of *P. vermiculatum* resulted in its complete oxidation into acid *III*.

Table 1. HPLC Parameters of Separation of Hydroxyfuniculosic (*III*) and Funiculosic (*IV*) Acids and Their Cyclization Products *V* and *VI*

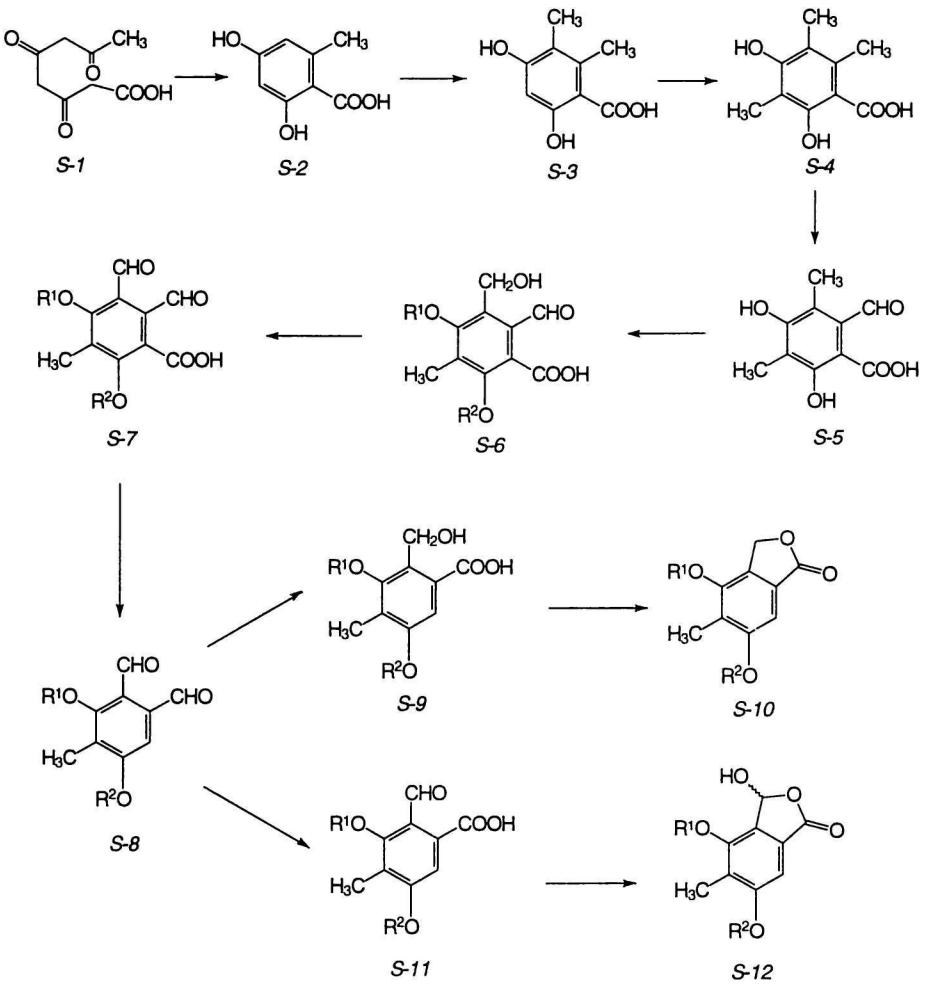
Compound	t_r/min	k'	N/m^{-1}	R
V	5.67	0.48	8200	2.50
VI	7.33	0.91	5000	2.20
III	9.17	1.39	7750	3.85
IV	13.33	2.48	6500	

t_r — retention time, k' — capacity factor, N — number of theoretical plates, R — resolution.

Table 2. Production of Acids *III* and *IV* in the Course of Cultivation of *P. vermiculatum*

t/h	ρ(Metabolite)/(mg cm^{-3})	
	III	*IV*
96	0.008	0.074
120	0.015	0.068
144	0.020	0.052
168	0.038	0.040

Acid *IV* is biosynthesized during the growth phase of the microorganism and is transformed into acid *III* in the phase in which the secondary metabolites are produced. There is a doubt about the mechanism of biosynthesis of compounds with arrangement of substituents as seen in the structure of acid *III* due to *meta* position of carboxyl group towards the hydroxyl groups [5]. We propose the mechanism of biosynthesis of this structure as follows: the primary precursor is the tetraketide (*S-1*, Scheme 1) condensed to orsellinic acid (*S-2*), which itself has been isolated from a separated lichen fungus [6] and has been detected in a



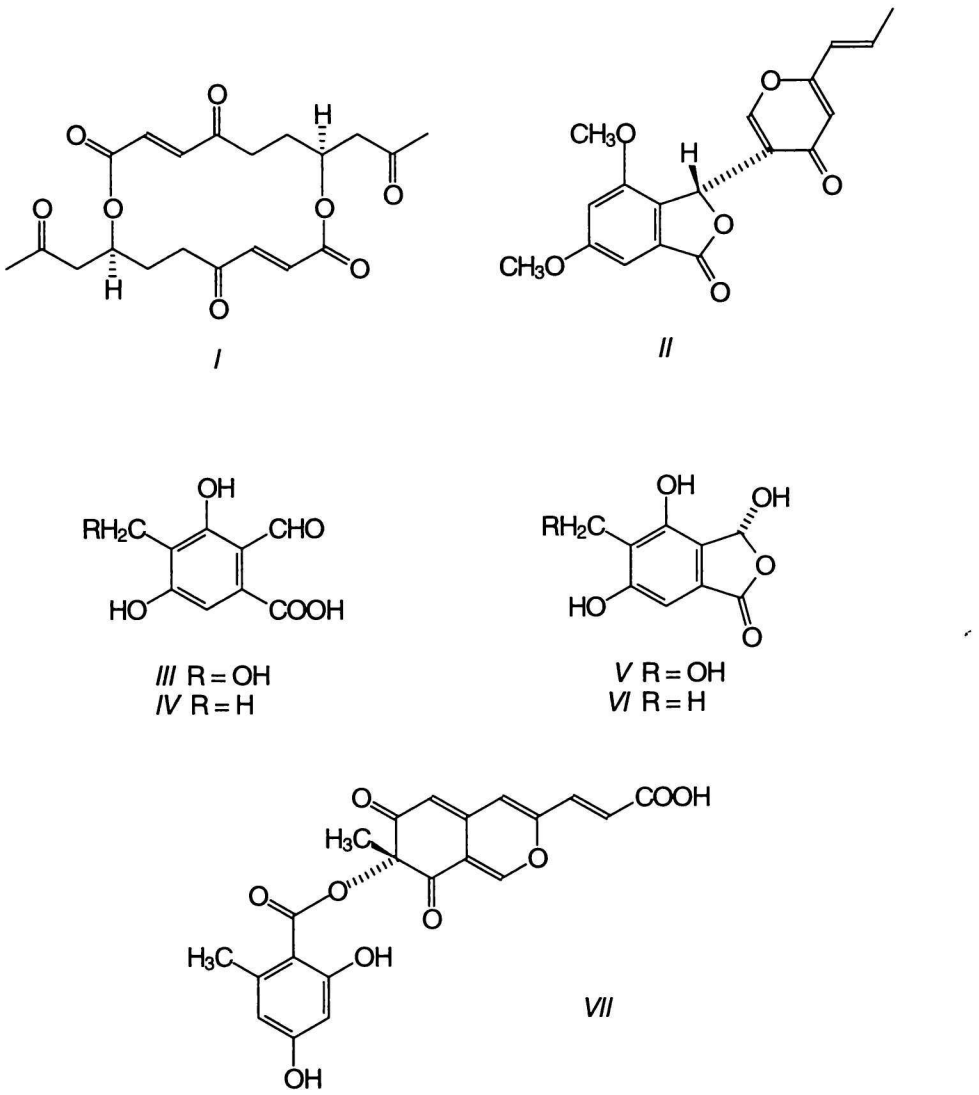
Scheme 1. Biosynthesis of phthalaldehydic acids.

number of fungi imperfecti. Acid *S-2* is rarely produced in high yield because it is easily transformed into *e.g.* lichen acids [7]. The further transformation of *S-2* proceeds *via* route outlined in Scheme 1 (the producing strain of the particular intermediate is given in the following text in parenthesis): methylation of acid *S-2* gives 4,6-dihydroxy-2,3-dimethylbenzoic acid (*S-3*, *Aspergillus terreus* [8] or *Gliocladium roseum* [9]) and 2,3,5-trimethyl-4,6-dihydroxybenzoic acid (*S-4*, *Mortierella ramanniana* [10]). Then, the regiospecific oxidation of the particular methyl groups proceeds affording cyclopolic (*S-6*, R^1 = H, R^2 = CH_3, *Penicillium cyclopium* [11]) and cyclopaldic acid (*S-7*, R^1 = H , R^2 = CH_3, *P. cyclopium* [12]). Decarboxylation of cyclopaldic acid analogue gives the quadrilineatin (*S-8*, R^1 = CH_3, R^2 = H, *A. quadrilineatus* [13]). Subsequent Canizzaro reaction of dialdehyde *S-8* would provide phthalide *S-10* (R^1 = H, R^2 = CH_3, *A. duricaulis* [5]; R^1 = R^2 = H, *Talaromyces flavus* [14]; R^1 = R^2 = CH_3, *A. silvaticus* [15]). Oxidation of dialdehyde *S-8* afforded funiculosic acid (*IV*/*S-11*, R^1 = R^2 = H, *P. funiculosum* [16], *P. vermiculatum* [4]).

P. vermiculatum, cultivated on glucose medium, produced vermistatin as the major metabolite. Mutants PV-1 and PV-2 biosynthesized on this medium (-)-mitorubrinic acid (*VII*) together with vermistatin (*II*) [17]. However, mutant PV-3 produced acids *III* and *IV* on this medium; *II* and *VII* were identified as the minor metabolites only. This is not surprising, because phthalaldehyde moieties are incorporated into structures of vermistatin (phthalide part) as well as of (-)-mitorubrinic acid (benzopyran grouping).

EXPERIMENTAL

Melting points were determined on a Kofler micro hot-stage, the UV spectra were measured with Specord 40M (Zeiss, Jena) spectrophotometer, ^1H and ^{13}C NMR spectra were recorded with a Varian model VXR-300 spectrometer at 300 MHz and 75 MHz, respectively. HPLC equipment (Laboratory Instruments, Prague) comprised an HPP 5001 pump, LCI 30 injector, LCD 2040 UV detector and CI-105 integrator; column: 250 mm × 4.6 mm, packed with



Formula 1

LiChrosorb RP-8, 7 μm (Merck, Darmstadt); mobile phase: methanol—water (φ_r = 55:45, pH 3 adjusted with H_3PO_4); flow rate: 0.7 cm^3 min^{-1}; wavelength of the UV detector: 230 nm. For TLC plates Silufol UV-254 (Kavalier, Votice, CR) were used in the system chloroform—methanol (φ_r = 9:1) visualized at λ = 254 nm, or by spraying with $FeCl_3$ solution.

Penicillium vermiculatum DANG. CCM F-276 and its mutants PV-1—PV-3 were used for cultivation. The medium CD-1 for submerged cultivation of *P. vermiculatum* was composed of (ρ/(g dm^{-3})): saccharose (90), $NaNO_3$ (2), KH_2PO_4 (1), KCl (1), $MgSO_4 \cdot 7H_2O$ (0.5), $FeSO_4 \cdot 7H_2O$ (0.01), tap water up to 1 dm^3, pH 6.3. In the medium CD-2 glucose replaced saccharose. These media were inoculated with a 10 vol.% of 24 h old inoculum and cultivated for 7 d at 28 °C.

Isolation of Metabolites

Cultivation broth (1.5 dm^3) was centrifuged, supernatant (1.1 dm^3) was extracted with ethyl acetate (three times, 250 cm^3 each), organic layers were combined, dried and concentrated. The residue was chromatographed on a silica gel packed column by a gradient elution with chloroform—methanol. The individual fractions were monitored by TLC. The combined fractions revealing R_f = 0.44 were crystallized from ether—methanol to yield acid *IV* (10 mg). Combined fractions of R_f = 0.24 were concentrated and crystal-

lized from toluene—methanol to furnish acid *III* (60 mg). Both acids were identified according to physicochemical, UV, MS, and NMR data [3].

Determination of Metabolites *III* and *IV* in Cultivation Medium

Cultivation medium (3.00 g) was thoroughly mixed with ethyl acetate (2.0 cm^3, 5 min), suspension was centrifuged (10 000 min^{-1}, 3 min), and 7 mm^3 of the supernatant were injected onto the chromatographic column. A linear relationship between peak area and mass concentration of the determined compounds in the range of 10—500 mg cm^{-3} was observed with the regression coefficient $r = 0.989$ for both compounds. Results were calculated for $n = 5$, $\alpha = 0.05$.

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