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EXPERIMENTAL ARTICLES

The Alkaloids of *Penicillium aurantiogriseum* Dierckx (1901) var. *aurantiogriseum* VKM F-1298

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Abstract—The fungus *Penicillium aurantiogriseum* var. *aurantiogriseum* VKM F-1298 produces two benzodiazepine alkaloids (anacine and aurantine) and one diketopiperazine alkaloid (aurantiamine). When cultured in a submerged mode in Abe medium, the alkaloids are mostly secreted into the medium. The dynamics of aurantine and aurantiamine accumulation in the medium is characterized by the presence of a relatively sharp maximum in the idiophase, whereas the accumulation of anacine in the medium is characterized by an extended plateau and occurs concurrently with fungal growth.

Key words: Penicillium aurantiogriseum, alkaloids, anacine, aurantine, aurantiamine.

Penicillium aurantiogriseum is one of the most widespread causal fungi of cereal diseases [1]. The study of foodstuffs in the endemic nephropathy area of Yugoslavia has led to the isolation of *P. aurantiogriseum* strains producing a water-soluble nephrotoxin [2, 3]. The nephrotoxic effect of these isolates, as well as of *Penicillium commune* strains isolated from moldy corn in Yugoslavia, was found to correlate with the production of aurantine and other benzodiazepine alkaloids [4], among which a new alkaloid, anacine, was discovered [5].

The aim of this work was to study the profile and biosynthesis of secondary metabolites of alkaloid origin in the fungus *P. aurantiogriseum* var. *aurantiogriseum* VKM F-1298, which was isolated from corn roots in Khmel'nitskaya oblast, Ukraine.

MATERIALS AND METHODS

The fungus *Penicillium aurantiogriseum* Dierckx (1901) var. *aurantiogriseum* VKM F-1298 was obtained from the All-Russia Collection of Microorganisms (VKM). The fungus was grown at 24 ± 1°C in 750-ml Erlenmeyer flasks with 100 ml of Abe medium containing (g/l) mannitol, 50.0; succinic acid, 5.4; $MgSO_4 \cdot 7H_2O$, 0.3; and KH_2PO_4, 1.0. The pH of the medium was adjusted to 5.2 with 25% NH_4OH. The medium was prepared by using tap water, distilled water, or distilled water supplemented with trace elements [6]. These elements were added to the medium as a sterile solution to give the following final concentrations (mg/l): $FeSO_4 \cdot 7H_2O$, 5.0; $ZnSO_4 \cdot 7H_2O$, 4.4; $MnSO_4 \cdot 5H_2O$, 1.7; $CuSO_4 \cdot 5H_2O$, 3.3; and Na_2MoO_4, 2.4. In some experiments, the fungus was grown in Czapek–Dox medium with 0.5% yeast extract [5]. The fungus was maintained on malt extract agar slants. The medium was inoculated with fungal spores. Cultivation was performed either on a shaker (220 rpm) or under stationary conditions.

Secondary metabolites were isolated from the culture liquid filtrate and from the mycelium. In the first case, the pH of the filtrate was adjusted to 7–8 with ammonia and secondary metabolites were extracted twice with ethylacetate. The extracts were pooled, dehydrated with anhydrous Na_2SO_4, filtered, and evaporated to dryness in a vacuum rotary evaporator. In the second case, secondary metabolites were isolated in three ways: (1) by twice extracting wet mycelium with a chloroform–methanol (2 : 1, v/v) mixture [7]; (2) by extracting lyophilized mycelium with acetone [5]; and by extracting (with ethylacetate) mycelium that was homogenized in an MPW-302 homogenizer (Mechanika precyzyjna, Poland).

The extracts were qualitatively analyzed by thin-layer chromatography (TLC) on Silufol UV-254 plates (Kavalier, Czech Republic) developed in solvent systems designated I, II, or III (mixtures of chloroform, methanol, and 25% NH_4OH in proportions of 90 : 10 : 1, 90 : 10 : 0.1, and 80 : 20 : 0.2, respectively).

The same extracts were quantitatively analyzed by HPLC using an LKB-Pribori AB liquid chromatograph equipped with a (150 cm × 4.6 mm) Nova-Pak C18 column (Waters, United States). The column was kept at 25°C. The eluted solutes were detected at 230 nm. The eluent was a methanol–water–25% NH_4OH (40 : 60 : 0.036 vol %) mixture with a flow rate of 0.9 ml/min. Measurements were carried out in triplicate. The stan-

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dard deviation ($n = 3$; $P = 0.95$) varied from 0.24 to 0.85 depending on the particular analyte.

To isolate secondary metabolites, the fungus was grown in Abe medium prepared in distilled water and supplemented with trace elements. The metabolites were fractionated by column chromatography on silica gel L 40/100 μm (Chemapol, Czech Republic). The column was developed with a gradient (100–80%) of a chloroform–methanol mixture containing 1% NH_4OH. The eluate was analyzed by TLC. The fractions that contained alkaloids were pooled and evaporated. Aurantiamine was purified by recrystallization from ethylacetate. Aurantine was purified by TLC on glass plates with silica gel LSL (Chemapol), which were developed in a chloroform–acetone (4 : 1) mixture. Anacine and its isomer were separated on plates with silica gel 60/Kieselguhr F-254 (Merck, Germany), which were developed in system I.

The absorption spectra of the metabolites dissolved in methanol were recorded using a UV-160A spectrophotometer (Shimadzu, Japan), and their mass spectra were obtained with a high-resolution Finnigan MAT 8430 mass spectrometer (Germany). Ionization was performed by electron impact (70 eV).

RESULTS AND DISCUSSION

The fungus *P. aurantiogriseum* belongs to the complex group of fungi of the genus *Penicillium*, which have similar morphological and physiological properties but differ in the profile of secondary metabolites produced [1, 8]. According to Pitt [3], *P. puberulum* Bainier (1907), *P. cyclopium* Westling (1911), *P. aurantiovirans* Biourge (1923), *P. lonoso-coeruleum* Thom (1930), and *P. verrucosum* var. *cyclopium* (Westling) Samson (1976) are synonyms of *P. aurantiogriseum* Dierckx (1901). The analysis of 519 isolates from the Northern Regional Research Laboratory collection that were preliminarily assigned to the species *P. cyclopium, P. viridicatum*, and *P. ochraceum* showed that they are very similar in micromorphology [1]. Nevertheless, some peculiarities of secondary metabolism, morphology, and physiology allowed nine new species (*P. aurantiogriseum, P. aurantiovirans, P. cyclopium, P. viridicatum, P. freii, P. melanoconidium, P. neoechinulatum, P. polonicum*, and *P. tricolor*) to be revealed among the isolates formerly assigned to the species *P. cyclopium* and *P. viridicatum.*

Moreover, a comprehensive analysis of morphological and chemotaxonomic features made it possible to distinguish several *P. aurantiogriseum* chemotypes: *P. aurantiogriseum* Dierckx (1901) var. *aurantiogriseum*; *P. aurantiogriseum* var. *melanoconidium* Frisvad, var. nov.; *P. aurantiogriseum* var. *neoechinulatum* Frisvad (1987), *P. aurantiogriseum* var. *polonicum* (Zaleski) Frisvad, comb. nov.; and *P. aurantiogriseum* var. *viridicatum* (Westling) Frisvad and Filtenborg, comb. nov. [8, 9]. The chemotype *P. aurantiogriseum* var. *aurantiogriseum* produces a wide range of secondary metabolites. Most of the isolates produce the mycotoxins penicillic acid, verrucosidin, xanthomegnin, vioxanthin, and viomellein, as well as aurantine, aurantiamine, and viridicatin alkaloids [8].

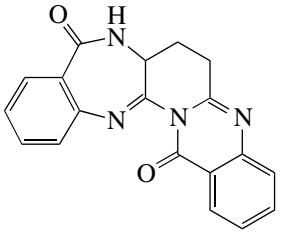
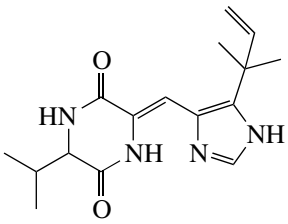
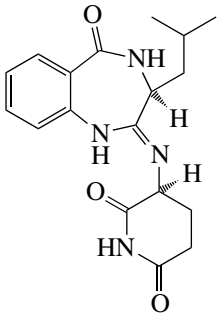
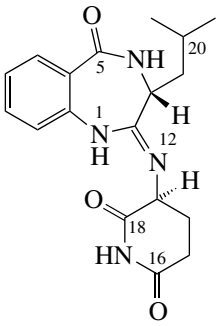
To study the profile of alkaloids produced by *P. aurantiogriseum* VKM F-1298, this strain was cultivated for 11 days in Abe medium in a submerged mode. Alkaloids were extracted from the culture liquid filtrate with ethylacetate. The total extract was fractionated by column chromatography on a column with silica gel, which was developed with a gradient (100–80%) of a chloroform–methanol mixture containing 1% NH_4OH. The eluate was analyzed by TLC in solvent systems I and II. As a result, four UV-absorbing fungal metabolites were isolated.

Metabolite **1**, with the highest mobility, was purified on a glass plate with silica gel LSL, which was developed in a chloroform–acetone (4 : 1) mixture. The UV spectrum of metabolite **1** had maxima at 228, 269, 279, 310, and 322 nm. The mass spectrum of this compound showed the presence of the $C_{19}H_{14}N_4O_2$ molecular ion, with a measured mass of 330.1115, and two intense fragments with $m/z = 277$ and 249, which were due to the successive elimination of $-C_3H_3N$ and $-CH_2N$ ions from the pyridodiazepine moiety of the molecule. This allowed metabolite **1** to be identified as aurantine, which was first isolated from *P. aurantiogriseum* IMI 180922 [10, 11]. Aurantine (6,7,7a,8-tetrahydroquinazole[3',2':1,6]pyrido[2,3-b][1,4]-benzodiazepine-9,16-dion) is a nephrotoxic alkaloid [4], whose molecule is composed of one glutamine and two anthranilic acid molecules (Table 1).

The secondmost mobile metabolite, **2**, was purified by recrystallization from ethylacetate. The UV spectrum of this compound had two typical diketopiperazine bands with absorption maxima at 231 and 320 nm [12]. The mass spectrum of metabolite **2** showed the presence of the $C_{16}H_{22}N_4O_2$ molecular ion, with a measured mass of 302.1745, and an intense fragment with m/z = 203, which was due to the elimination of the $-C_5H_9ON$ ion from the diketopiperazine ring of the molecule. These data allowed metabolite **2** to be identified as the cyclic dipeptide alkaloid aurantiamine, whose molecule contains a diketopiperazine ring composed of valine and histidine. This alkaloid was first isolated from *P. aurantiogriseum* var. *aurantiogriseum* IBT 3699-IBT [7]. Aurantine differs from the similar alkaloid viridiamine of *P. viridicatum* in a substituent occurring in the imidazole group of the molecule.

Two other metabolites, **3** and **4**, showed similar chromatographic mobilities on Silufol plates and could be separated only on Merck plates (Table 1). The UV spectrum of the major metabolite **3** had absorption bands at 226.5, 269.5, 276.5, 303.5, and 317.5 nm. The minor metabolite **4** had a similar UV spectrum with absorption maxima at 226.5, 267.0, 275.0, 304.0, and 316.5 nm. The mass spectra of metabolites **3** and **4** were similar and showed the presence of the $C_{18}H_{22}O_3N_4$

Table 1. The physicochemical characteristics of alkaloids isolated from *P. aurantiogriseum* var. *aurantiogriseum* VKM F-1298

Metabolite	Relative mobility ($R_f \times 100$) on Silufol in systems			Characteristic peaks in mass spectra, *m/z* (%)	Structure
	I	II	III		
1. Aurantine	47	59	–	M^+ 330(45), 290(5), 277(100), 249(91), 234(24), 220(16), 192(12), 130(12), 124(16), 102(10)	
2. Aurantiamine	32	38	57	M^+ 302(100), 287(21), 260(38), 231(32), 203(78), 188(46), 175(32), 160(48), 133(39), 135(19)	
3. Anacine	18*	27	48	M^+ 342(53), 325(46), 299(15), 286(100), 269(93), 254(21), 241(31), 227(74), 214(41), 198(22), 186(17), 185(29), 130(19)	
4. Isoanacine	20*	–	53	M^+ 342(100), 325(27), 299(15), 286(75), 282(30), 269(66), 254(27), 241(22), 227(56), 214(40), 198(21), 186(20), 185(17), 130(20)	

* The relative mobilities of anacine and isoanacine on Merck plates in this solvent system are 22 and 28, respectively.

molecular ion, with a measured mass of 342,1690, and fragments with equal masses but different intensities. The molecular ion was fragmented through the elimination of either $M^+–NH_3$ (*m/z* 325), the $M^+–C_6H_{13}ON$ fragment (*m/z* = 227) of the diazepine ring, or the $M^+–C_3H_7$ and $M^+–C_4H_8$ fragments (*m/z* = 299 and 286, respectively) of the isoprene substituent. The metabolites differed in the temperature of evaporation in the ionic source. Unexpectedly, the intensity of the molecular ion increased with a rise in the temperature of evaporation, which could be accounted for by the existence of two molecular forms, the less stable form being converted spontaneously into the more stable form. The major metabolite **3** was identified as anacine (a benzodiazepine alkaloid composed of anthranilic acid, L-leucine, and L-glutarimide), whereas the minor metabolite **4** was identified as an anacine isomer containing D-leucine instead of L-leucine. The proportion between anacine and isoanacine was about 10 : 1. Both anacine and isoanacine were first isolated from the *P. aurantiogriseum* strain recovered from moldy corn in the endemic nephropathy area of Yugoslavia [4, 5]. Anacine can eas-

Table 2. The content of alkaloids in the mycelium of *P. aurantiogriseum* var. *aurantiogriseum* VKM F-1298 grown under stationary conditions

Cultivation medium	Biomass, g/l	Alkaloids, mg/g		
		Aurantine	Aurantiamine	Anacine
Czapek–Dox	4.0	0.65	0.22	0.39
Abe (distilled water)	9.3	0.05	0.04	0.32
Abe (tap water)	19.2	0.46	0.60	9.20

Table 3. The content of alkaloids in the culture liquid filtrate of *P. aurantiogriseum* var. *aurantiogriseum* VKM F-1298 grown under stationary conditions

Cultivation conditions	Cultivation medium	Biomass, g/l	Aurantine		Aurantiamine		Anacine	
			mg/l	mg/g	mg/l	mg/g	mg/l	mg/g
11th day of submerged cultivation	Czapek–Dox	9.0	0.7	0.086	0.3	0.03	2.0	0.22
	Abe (distilled water)	6.7	15.9	2.34	19.5	2.91	24.9	3.72
	Abe (tap water)	21.8	24.8	1.14	17.0	0.78	10.7	0.47
	Abe (distilled water with trace elements)	20.0	9.2	0.46	13.9	0.69	28.6	1.43
18th day of stationary cultivation	Czapek–Dox	4.0	9.5	2.37	18.5	4.62	4.6	1.15
	Abe (distilled water)	9.3	1.0	0.11	0.6	0.07	0.8	0.09
	Abe (tap water)	19.2	7.5	0.39	7.0	0.36	3.4	0.18

ily isomerize to isoanacine upon heating. In addition to anacine and isoanacine, the *P. aurantiogriseum* strain produces the benzodiazepine alkaloids cyclopenin and cyclopenol and the diketopiperazine alkaloid fructigenine A [5].

It is known that the cultivation conditions of microscopic fungi may influence the amount and composition of their secondary metabolites [13]. To reveal the biosynthetic potential of strain VKM F-1298, it was cultivated in submerged and stationary modes on Czapek–Dox medium with 0.5% yeast extract [5, 7, 10, 11] and on Abe medium with two carbon sources (mannitol and succinic acid) [6]. Reportedly, the latter medium is optimal for the production of alkaloids by *Penicillium* fungi [14]. The media were prepared by using distilled water, tap water (this water often gives good results, although it is rarely used in such studies because of seasonal variations in the composition), and distilled water supplemented with Abe trace element mixture (this mixture is recommended by some researchers to enhance alkaloid synthesis [14, 15]).

In all cases, strain VKM F-1298 produced mainly aurantine, aurantiamine, and anacine. During submerged cultivation, the alkaloids were secreted into the medium. Aurantine and aurantiamine were found to be readily extracted from culture liquid filtrate with chloroform, whereas anacine is extracted with more polar solvents, such as ethylacetate.

To extract alkaloids from the mycelium, we applied methods that were proposed by other researchers—the extraction of lyophilized biomass with acetone [5] and the repeated extraction of wet mycelium with a chloroform–methanol (2 : 1) mixture [7]. In addition, we used our own method—the extraction of homogenized mycelium with ethylacetate. The mycelium of strain VKM F-1298 was homogenized in water. The homogenate was filtered, and the filtrate was extracted two times with ethylacetate. The mycelium cake formed on the filter was suspended in ethylacetate and filtered again. All the ethylacetate extracts were pooled, dehydrated with anhydrous Na_2SO_4, and evaporated to dryness. The latter method gave better results than the other methods. For instance, only this method allowed trace amounts of aurantine and aurantiamine to be detected in mycelium from the submerged culture.

The mycelium grown under stationary conditions showed the presence of all three alkaloids (Table 2), the greatest amount of alkaloids being present in the mycelium grown on Abe medium that was prepared with tap water. The growth of the mycelium on this medium was also the best. At the same time, the concentration of the alkaloids in the culture liquid and their relative content in the mycelium were maximal when the strain was grown on Czapek–Dox medium under stationary conditions (Table 3).

The Czapek–Dox medium used by Boyes-Korkis *et al.* [5] for the production of the three alkaloids turned out to be nonoptimal for the submerged cultivation of strain VKM F-1298. Moreover, the addition of $CaCl_2$ at concentrations of up to 2% (as recommended by Boyes-Korkis *et al.*) to Czapek–Dox medium with

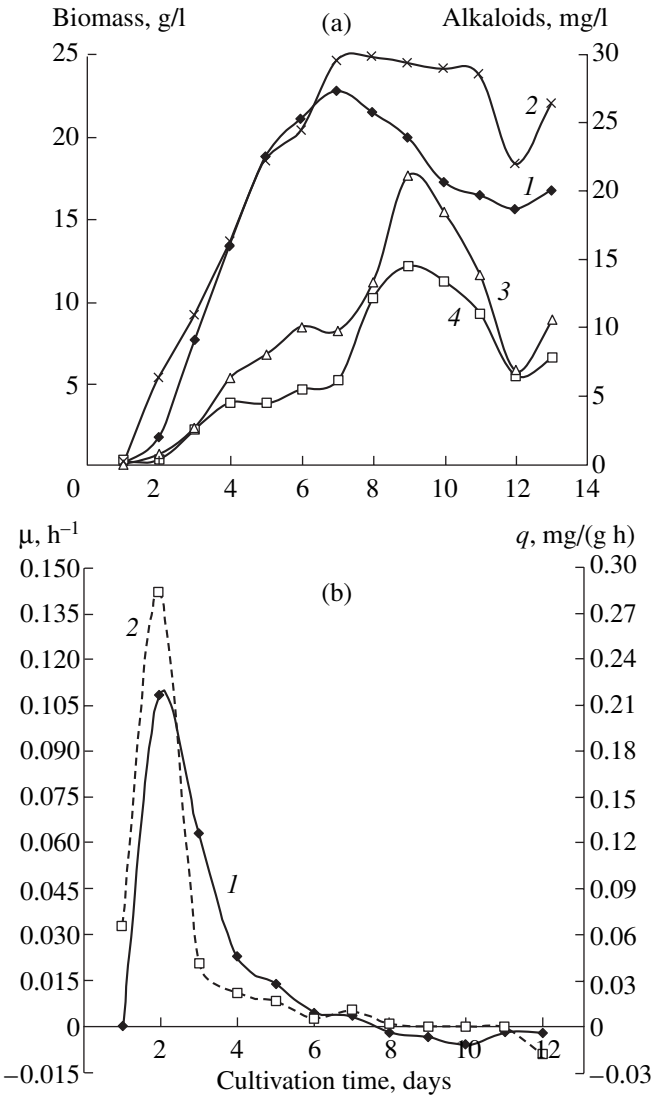


Fig. 1. The growth parameters of strain VKM F-1298 grown in a submerged mode in Abe medium with trace elements. (a) (*1*) Biomass and (*2–4*) the concentration of (*2*) anacine, (*3*) aurantiamine, and (*4*) aurantine. (b) (*1*) Specific growth rate (μ) and (*2*) the specific rate of anacine accumulation (*q*) in the culture liquid.

yeast extract suppressed the growth of strain VKM F-1298.

The submerged cultivation of strain VKM F-1298 in the Abe medium that was prepared with distilled water provided for the maximum alkaloid synthesis under submerged conditions, although fungal growth was poor in this case (Table 3). The use of tap water and the addition of trace elements to Abe medium greatly increased the mycelium biomass but decreased the productivity of alkaloid synthesis (Table 3).

Boyes-Korkis *et al.* [5] found that the biomass of *P. aurantiogriseum* IMI 357488 cultivated in a 60-l fermentor reached a maximum by the 42nd hour of cultivation, the lysis of the culture lasted six days, and the production of anacine reached a maximum on the fourth day of cultivation (in the stationary growth phase).

To study the relationship between the growth and alkaloid synthesis in strain VKM F-1298, it was cultivated in a submerged mode in Abe medium prepared with distilled water and supplemented with trace elements. The maximum biomass (~23 g/l) was observed on the seventh day of cultivation, after which it tended to decrease without forming a plateau (Fig. 1). The absence of a distinct stationary growth phase can be accounted for by the action of autolysis inducers. The specific growth rate of the fungus was high ($\mu = 0.1\ h^{-1}$) as compared to other representatives of the genus *Penicillium*.

The benzodiazepine alkaloid anacine was synthesized by strain VKM F-1298 in parallel to its growth (Fig. 1). Namely, anacine was synthesized beginning from the first day of cultivation and its concentration in the medium reached a maximum (up to 30 mg/l) concurrently with the biomass. It should be noted that the maximum concentration of anacine persisted for several days.

The other benzodiazepine alkaloid, aurantine, was synthesized in a different manner. Actually, the dynamics of aurantine synthesis resembled that of the diketopiperazine alkaloid aurantiamine (Fig. 1). The production of these two alkaloids reached a maximum in the phase of partial lysis (on the eighth to ninth day of cultivation) and then drastically decreased, likely due to their high metabolic activity.

Thus, the fungus *Penicillium aurantiogriseum* var. *aurantiogriseum* VKM F-1298 produces two benzodiazepine alkaloids (anacine and aurantine) and one diketopiperazine alkaloid (aurantiamine). When cultured in a submerged mode in Abe medium (this medium is the most beneficial for alkaloid synthesis), the alkaloids are mostly secreted into the medium. The dynamics of aurantine and aurantiamine accumulation in the Abe medium prepared with distilled water and supplemented with trace elements is characterized by the presence of a relatively sharp maximum in the idiophase, whereas the accumulation of anacine in this medium is characterized by an extended plateau and occurs concurrently with fungal growth.

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