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Synthesis of α-Cyclopiazonic Acid by Fungi of the Genus *Aspergillus*

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Abstract—The presence of α-cyclopiazonic acid has been studied among metabolites of *Aspergillus* fungi. The study was performed with 138 cultures of 13 species obtained from the All-Russia Collection of Microorganisms and the collection of our institute. α-Cyclopiazonic acid was most frequently encountered among the metabolites of the section *Flavi* (the ability to synthesize α-cyclopiazonic acid was expressed in 61% of the strains of *A. flavus*, 83% of the strains of *A. oryzae*, and all strains of *A. tamarii*). This expression index for *A. versicolor* was less than 5%. We showed for the first time that α-cyclopiazonic acid is produced by *A. fumigatus* and *A. phoenicis* (expression in 30% of the strains of either species).

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α-Cyclopiazonic acid (CPA) is a mycotoxin. It is formed biogenetically from the molecules of tryptophan and mevalonic acid (one of each) and two molecules of acetic acid. CPA was first described as a toxic metabolite of *Penicillium cyclopium* in 1968 [1]. CPA is a well-studied compound. The structure, pathways of biosynthesis, and several enzymes for the biosynthesis of CPA have been described [2, 3].

CPA exhibits high neurotoxic activity (causing muscle tremors and seizures with lethal outcomes) and induces malignant tumors [4, 5]. The type of liver damage by CPA differs from hepatocarcinogenesis induced by aflatoxins and sterigmatocystin [6].

CPA is a natural contaminant of food products and fodder (e.g., cheeses, corn, nuts, cereals, and meat) [5–7]. This mycotoxin is produced by fungi used for manufacturing enzyme products and drinks [8].

CPA is a secondary metabolite of the fungi of genera *Penicillium* and *Aspergillus*. The following fungal species of the first genus produce CPA: *P. aurantiogriseum* (=*P. cyclopium* and =*P. puberulum*), *P. camemberti, P. solitum* var. *solitum* (=*P. patulum*), *P. solitum* var. *crustosum* (=*P. crustosum*), and *P. viridicatum* [5–9]. The highest occurrence of the ability to produce CPA (expression index) was demonstrated for *P. camemberti* (68 of the 69 test strains), *P. commune* (132 of the 132 test strains), *P. griseofulvum* (40 of the 40 test strains), and *P. glandicola* var. *mononematosum* (=*P. mononematosum*, 49 of the 49 test strains) [10]. *P. vulpinum* (three of the nine test strains) [11] and *P. duclauxii* (*P. clavigerum*, one of the two test strains) [12] are CPA-producing species. According to R.M. Scott [6], CPA is produced by all isolates of *P. camemberti* from cheese (63 of the 63 test strains). The species with a high occurrence of CPA synthesis also includes *P. aurantiogriseum* (*P. cyclopium*, seven of the seven test strains; and *P. puberulum*, eight of the eight test strains) [13].

Little is known about CPA-producing fungi of the genus *Aspergillus*. The species *A. flavus* is a well-known producer of the most hazardous mycotoxins (aflatoxins). B.W. Horn and J.W. Dorner revealed CPA in 83% of the strains of *A. flavus* isolated from soil samples of various regions of the United States [14]. CPA is produced by 22 of the 23 *A. tamarii* cultures [9]. Individual strains of *A. versicolor* and *A. oryzae* also produce CPA [8, 15, 16]. Experiments performed by Hermansen *et al.* with three strains of *A. versicolor* and four strains of *A. oryzae* detected CPA synthesis only in two strains of *A. oryzae* [13].

This work was designed to evaluate the CPA-producing capacity of *Aspergillus* fungi.

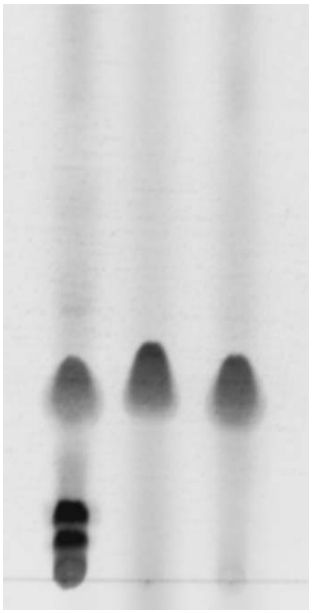
MATERIALS AND METHODS

Object of study. The study was performed with 138 fungal cultures of the genus *Aspergillus* (13 species, five sections), obtained from the All-Russia Collection of Microorganisms (VKM) and the collection of our institute. These fungi were isolated from various habitats.

Section *Clavati*. *A. clavatus* Desmazieres – VKM F-22, VKM F-738, VKM F-802, VKM F-1330, VKM F-1594, and VKM F-2608; and *A. giganteus* Wehmer – VKM F-29.

Section *Fumigati*. *A. fumigatus* Fresenius – Ff-1, Ff-3, Ff-9, Ff-11, Ff-17, Ff-18, Ff-24, Ff-25, Ff-33, Ff-44, Ff-48, Ff-85, and Ff-141.

Section *Flavi*. *A. flavus* Link – VKM F-1024, Fl-1, Fl-2, Fl-3, Fl-6, Fl-11, Fl-12, Fl-13, Fl-14, Fl-20, Fl-23,



Chromatogram of extracts from the culture liquid of *Aspergillus oryzae* VKM F-50 obtained at various pH values. (*1*) Acidic extracts (pH 2–3), (*2*) CPA standard, and (*3*) alkaline extracts (pH 8–9).

Fl-24, Fl-31, Fl-44, Fl-57, Fl-58, Fl-63, and Fl-132; *A. oryzae* (Ahlburg) Cohn – VKM F-45, VKM F-47, VKM F-49, VKM F-50, VKM F-51, VKM F-53, VKM F-55, VKM F-56, VKM F-71, VKM F-763, VKM F-2094, VKM F-2095, VKM F-2096, VKM F-2097, VKM FW-54, VKM FW-609, and VKM F-2419; and *A. tamarii* Kita – VKM F-64, VKM FW-2704, and VKM FW-2711.

Section *Nigri*. *A. aculeatus* Iizuka – VKM FW-2415, VKM FW-2416, VKM FW-2417, and VKM FW-2418; *A. awamori* Nakazawa – VKM FW-746, VKM F-758, and VKM F-808; *A. carbonarius* (Bainier) Thom – VKM F-21; *A. japonicus* Saito – VKM F-2145, *A. niger* van Tieghem – VKM FW-395, VKM FW-650, VKM FW-848, VKM FW-2532, VKM FW-2555, VKM FW-2556, VKM FW-2557, VKM FW-2558, VKM FW-2743, VKM FW-2744, VKM FW-2745, VKM FW-2746, VKM FW-2747, VKM FW-2748, VKM FW-2749, VKM FW-2750, VKM FW-2751, Fn-71, and Fn-72; and *A. phoenicis* (Corda) Thom et Currie – VKM F-2084, Fp-1, Fp-4, Fp-5, Fp-7, Fp-9, Fp-10, Fp-14, Fp-15, Fp-18, Fp-23, Fp-25, Fp-27, Fp-29, Fp-31, Fp-39, Fp-41, Fp-51, Fp-52, Fp-55, Fp-65, Fp-71, Fp-75, Fp-101, Fp-102, Fp-136, Fp-138, Fp-150, Fp-250, and Fp-650.

Section *Versicolores*. *A. versicolor* (Vuillemin) Tiraboschi – VKM F-70, VKM F-804, VKM F-1114, VKM F-2546, VKM F-2993, VKM FW-438, VKM FW-447, VKM FW-681, VKM FW-722, VKM FW-916, VKM FW-2023, VKM FW-2752, Fv-1, Fv-2, Fv-3, Fv-4, Fv-5, Fv-20, Fv-40, Fv-50, and Fv-80.

Cultivation. The micromycetes were cultured in a medium containing (per 1 l distilled water) 50.0 g mannitol, 5.4 g succinic acid, 0.3 g $MgSO_4 \cdot 7H_2O$, and 1.0 g KH_2PO_4; pH was brought to 5.4 with 25% NH_4OH. Microelements were added before inoculation. We used the following microelements: 5.0 mg/l $FeSO_4 \cdot 7H_2O$, 3.3 mg/l $CuSO_4 \cdot 5H_2O$, 4.4 mg/l $ZnSO_4 \cdot 7H_2O$, 2.7 mg/l $MnSO_4 \cdot 5H_2O$, and 2.4 mg/l Na_2MoO_4. Inoculation was performed using an aqueous suspension of 7-day-old cultures grown in tubes with malt agar (3 vol %). Cultivation was performed in 750-ml Erlenmeyer flasks (each containing 150 ml of the medium) at 26°C and 180–200 rpm.

Isolation and study. CPA was isolated on days 6 and 12 of growth. The culture liquid filtrate (100 ml) was alkalized to pH 8–9 with 25% NH_4OH. CPA was extracted with chloroform. The extract was desiccated using anhydrous Na_2SO_4, filtered, and evaporated to dryness on a rotary evaporator at a temperature of no more than 40°C.

The extract was studied by TLC on UF-254 Silufol plates (Czech Republic) in a solvent system containing ethyl acetate, methanol, and 25% ammonia (85 : 15 : 10). The sample was diluted with 100 μl of a chloroform–methanol mixture (1 : 1). An aliquot of the solution (4–8 μl) and a standard were loaded on a Silufol plate. CPA from *Penicillium palitans* VKM F-3088 served as a standard [17]. The metabolites were detected on the plates by UV absorption or by spraying with the Ehrlich reagent. CPA was identified by its chromatographic mobility, staining with the Ehrlich reagent, and the shape of the spot.

RESULTS AND DISCUSSION

Modern methods of CPA study include HPLC, TLC, enzyme immunoassay, and spectrophotometry [8]. TLC on silica gel treated with oxalic acid is the most reliable method of CPA analysis [8, 13]. This simple and readily available method is complemented by a color reaction with the Ehrlich reagent.

In most systems is detected on silica gel not treated with oxalic acid, CPA as a long and blurry spot. This feature significantly decreases the sensitivity of the method. We showed that the use of a strongly polar system containing ethyl acetate, methanol, and 25% ammonia (85 : 15 : 10) yields good results during TLC on plates not impregnated with oxalic acid. CPA formed a compact spot with a slightly sharpened apex. The sensitivity of the measurement was 0.5 μg.

CPA is an amphoteric compound. Therefore, CPA may be extracted at various values of pH. The culture liquid contained considerable amounts of acidic side products. Hence, we proposed to perform extraction at a slightly alkaline pH (Figure).

We examined 138 cultures of *Aspergillus* fungi from diverse habitats (soil samples of various regions, arctic permafrost soil, raw plant materials, and leather goods).

CPA-producing micromycetes of the genus *Aspergillus*

Species	Number of test strains	Number of CPA-producing fungi	List of CPA-producing fungi	Expression index
			Section *Clavati*	
A. clavatus	6	0		0
A. giganteus	1	0		0
			Section *Fumigati*	
A. fumigatus	13	4	Ff-9, Ff-11, Ff-33, Ff-141	31
			Section *Flavi*	
A. flavus	18	11	Fl-1, Fl-2, Fl-6, Fl-11, Fl-12, Fl-13, Fl-23, Fl-24, Fl-58, Fl-63, Fl-132	61
A. oryzae	18	15	VKM F-45, VKM F-47, VKM F-49, VKM F-50, VKM F-51, VKM F-52, VKM F-55, VKM F-56, VKM F-71, VKM F-763, VKM F-2094, VKM F-2095, VKM F-2096, VKM FW-54, VKM FW-609	83
A. tamarii	3	3	VKM F-64, VKM FW-2704, VKM FW-2711	100
			Section *Nigri*	
A. aculeatus	4	0		0
A. awamori	3	0		0
A. carbonarius	1	0		0
A. japonicus	1	0		0
A. niger	19	0		0
A. phoenicis	30	9	Fp-1, Fp-15, Fp-18, Fp-23, Fp-31, Fp-39, Fp-51, Fp-71, Fp-75	30
			Section *Versicolores*	
A. versicolor	21	1	Fv-1	>5

The taxonomic diversity of the sampling was represented by 13 species belonging to five sections. The species of the sections *Clavati* and *Fumigati* are uniseriate. The fungi of the sections *Flavi* and *Nigri* uniseriate or biseptate (sometimes even within the same strain). The species of the section *Versicolores* are biseptate.

Our study showed that the micromycetes of the section *Flavi* are most potent in producing CPA (Table 1). This toxin was detected in 15 of the 18 cultures of *A. oryzae*. The expression index was high for *A. flavus* (11 of the 18 strains). *A. tamarii* strains also produced CPA (three of the three strains). The fungi of this section are widespread in nature. They inhabit soils of subtropical and tropical regions. Moreover, these micromycetes colonize food and feed substrates (e.g., peanut, corn, cereals, legumes, cotton plant seeds, several fruits, vegetables, spicery, and fodder) [4, 7, 9].

A. versicolor is typically distributed in soil samples of the temperate zone and may be isolated from permafrost soil. The fungus is widely known as a degrader of industrial materials, including optical and radio devices. The strains of this species are isolated from fruits and produce various enzymes and toxins [18]. In 1973, *A. versicolor* was shown to synthesize CPA, its imine, and β-CPA [16]. Since then, it is considered as a CPA producer [5, 6]. However, we demonstrated that the ability to synthesize CPA is not as typical of *A. versicolor* as many authors believe. This metabolite was detected in only 1 of the 21 strains of this species isolated from the soil of the Aleutian Islands (Fv-1 isolate). Hermansen *et al.* did not find CPA producers among the three strains of *A. versicolor* studied [13].

The species *A. fumigatus* is one of the most toxigenic food contaminants. Four of the thirteen isolates of *A. fumigatus* were shown to synthesize CPA. We were, therefore, the first to demonstrate the presence of CPA in *A. fumigatus*.

Among the micromycetes of the section *Nigri*, we studied the strains of six species. The study was performed with 60 micromycetes. Most of them belong to the morphologically similar species of *A. niger* and *A. phoenicis*. The published data show that these species may be considered as variants of the species *A. niger* (*A. niger* var. *niger* and *A. niger* var. *phoenicis*, respectively) [19]. CPA synthesis was found only in strains of *A. phoenicis*. The expression index was 30%. The ability to synthesize CPA was not detected in *A. niger* strains, which showed no dependence on the substrate of isolation (soil, permafrost soil, or plant material) and the time of storage (from 1 month to 10 years). The mycotoxin was also not detected in other species of this section *(A. aculeatus*, *A. awamori*, *A. carbonarius*, and *A. japonicus*).

The strains of the section *Clavati* are typical contaminants of plant residues and food products. They are pathogenic for fishes and animals [18]. Seven specimens of section *Clavati* (*A. clavatus* and *A. giganteus*) did not synthesize CPA.

Our results show that, among 138 cultures of the 13 species, the highest number of CPA-producing fungi was found in micromycetes of the section *Flavi* (*A. flavus*, 61%; *A. oryzae*, 83%; *A. tamarii*, 100%). The expression index for *A. versicolor* fungi was less than 5%. We showed for the first time that CPA is produced by *A. fumigatus* and *A. phoenicis* (the expression index was equal to 30% for each species).

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