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Occurrence of Indole Alkaloids among Secondary Metabolites of Soil *Aspergillus* Spp.

N. G. Vinokurova, I. I. Khmel'nitskaya, B. P. Baskunov, and M. U. Arinbasarov

Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushchino, Moscow oblast, 142290 Russia
e-mail: arin@ibpm.serpukhov.su

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Abstract—The occurrence of indole alkaloids among secondary fungal metabolites was studied in species of the genus *Aspergillus*, isolated from soils that were sampled in various regions of Russia (a total of 102 isolates of the species *A. niger, A. phoenicis, A. fumigatus, A. flavus, A. versicolor, A. ustus, A. clavatus*, and *A. ochraceus*). Clavine alkaloids were represented by fumigaclavine B, which was formed by *A. fumigatus*. α-Cyclopiazonic acid was formed by isolates of *A. fumigatus, A. flavus, A. versicolor, A. phoenicis*, and *A. clavatus*. The occurrence of indole-containing diketopiperazine alkaloids was documented for isolates of *A. flavus, A. fumigatus, A. clavatus*, and *A. ochraceus*. No indole-containing metabolites were found among the metabolites of *A. ustus* or *A. niger*.

Microscopic fungi of the genus *Aspergillus* are typical microbial soil saprophytes. They are known to produce highly toxic secondary metabolites [1]. Toxic metabolites of *Aspergillus* form and accumulate in soil, cause toxic damage, and inhibit plant growth via the soil [2]. Although soil is the main habitat of *Aspergillus* fungi, they can grow on various substrates and are sometimes abundant in grain, its processing products, and industrial substrates. Fodders and foodstuffs infected by *Aspergillus* cause aspergillosis and aspergillotoxicosis in man, domestic animals, and birds [1, 3]. In addition, toxins of *Aspergillus* are known to be toxic to plants [2]. Extracts from culture liquid and mycelium cause fading of cuttings and seedlings, canker, constriction of plant vessels, etc. Infection of soil with some toxin-producing fungi results in reduction of germination and retardation of seedling growth.

The most common and best investigated fungal toxins are those formed by the polyketide acetate conversion pathway, involving relatively few simple intermediates of basal metabolism. They include, first of all, aflatoxins (*A. flavus* and *A. parasiticus*), ochratoxins (*A. ochraceus*), sterigmatocystin (*A. versicolor* and *A. nidulans*), citrinin (*A. terreus*, *A. candidus*, and *A. niveus*), and patulin (*A. terreus*) [1]. The investigation of these toxins and their producers, which are natural contaminants of foodstuffs and fodders, concerns environmental protection and the health of man and animals [3]. Less often, fungi of the genus *Aspergillus* are investigated with regard to alkaloids derived from amino acids. Exceptions are *A. fumigatus*, which produces a large set of fumitremorgin alkaloids, and *A. clavatus*, which produces tryptoquivalines [4].

In contrast, micromycetes of the genera *Claviceps* and *Penicillium* are known mainly as alkaloid producers [5]. The species dependence of their alkaloid pattern was closely investigated for fungi of the genus *Penicillium* [6].

The goal of this study was to investigate the occurrence of indole alkaloids among secondary metabolites of soil-inhabiting *Aspergillus* isolates.

METHODS

Experiments were performed with strains isolated from soils of various regions of Russia: the Aleutian Islands (A), Alupka (Al), the White Sea biological station of Moscow State University (WSS), the Kara-Dag reserve (Kg, forest area; Kd, mountain slope), Pushchino (B, birch forest; As, aspen forest), Foros (F), Samara oblast (Rs, ruderal steppe areas; Chs, Chubov steppe), Simeiz (S), Tver oblast (T), the Teberda reserve (Td), and Tomsk oblast (To). The fungi were isolated in 1999–2001 and identified by conventional methods [7]. A total of 102 isolates were studied.

The fungi were cultivated on Czapek slant agar. Inocula were grown for 3–5 days on modified Abe medium [8, 9]. Ten milliliters of inoculum were used for the next step.

The strains were grown by submerged cultivation at 24 ± 1°C in 750-ml flasks with 150 ml of the same medium, shaken at 180–200 rpm. Metabolites were isolated from the filtered culture liquids after 6 and 12 days of growth. The filtrates were alkalized with 25% ammonia to pH 8–9 and extracted three times with chloroform. The extracts were dried with anhydrous Na_2SO_4, filtered and evaporated to dryness.

Analysis of the extracts and preparative isolation of alkaloids were carried out using TLC, on Silufol UV-254 plates (Czech Republic) using the mixtures (I) chloroform–methanol–25% ammonia (90 : 10 : 1),

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(II) the same solvents (90 : 10 : 0.1), (III) chloroform–acetone (9 : 1), or (IV) ethyl acetate–toluene–acetic acid (5 : 4 : 1). Metabolites were detected by UV absorption and after treatment with Ehrlich reagent. Individual zones were eluted with methanol, evaporated, and analyzed by physicochemical methods.

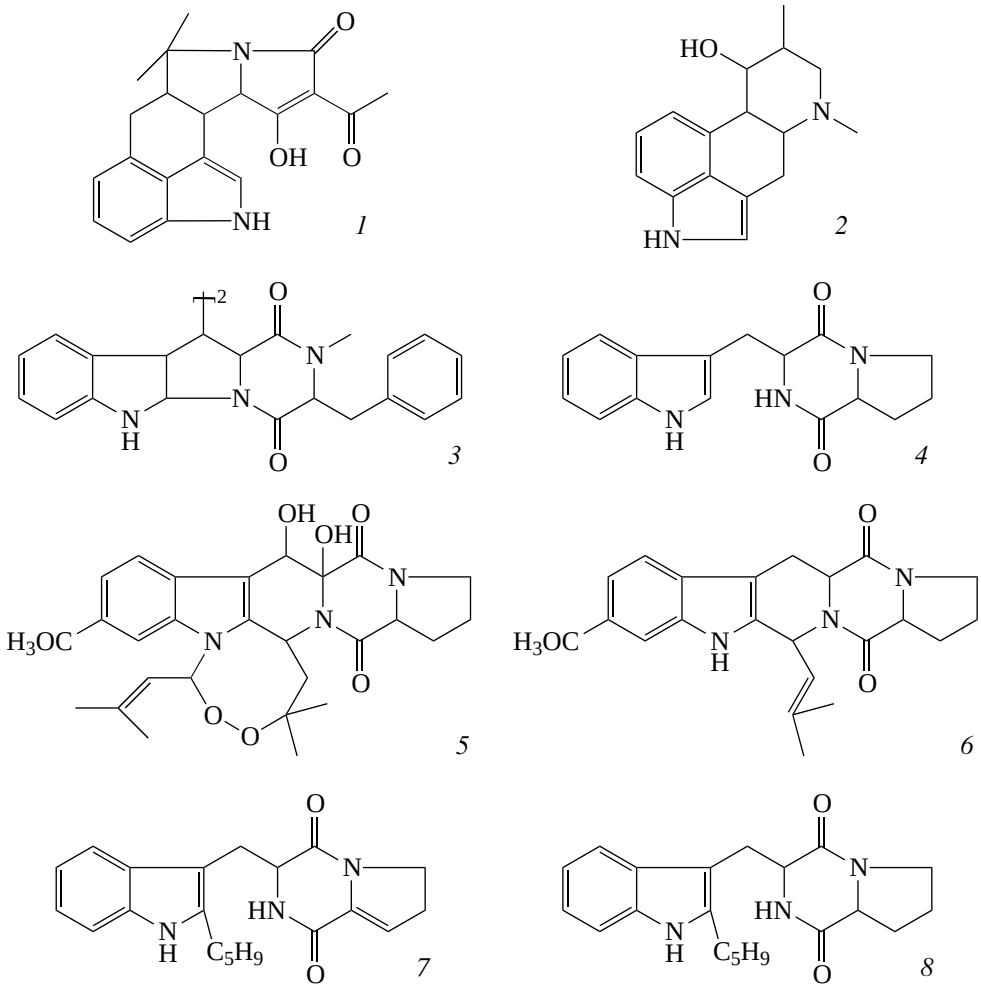
Metabolites were identified using TLC, UV spectrometry, mass spectrometry, and indole-specific Ehrlich reagent. UV spectra of the metabolites were recorded in methanol solutions on a Shimadzu UV-160A spectrophotometer (Japan). Mass spectra were recorded on a Finnigan MAT 8430 high-resolution mass spectrometer (Germany) at an ionizing-electron energy of 70 eV.

RESULTS AND DISCUSSION

The following *Aspergillus* species were investigated (number of isolates): *A. niger* (2), *A. phoenicis* (30), *A. fumigatus* (13), *A. flavus* (19), *A. versicolor* (9), *A. ustus* (19), *A. clavatus* (1), and *A. ochraceus* (9).

As demonstrated in numerous papers, the best medium for producing alkaloids of various structures by species of *Penicillium* is the modified Abe medium containing two carbon sources (mannitol and succinate) [10, 11]. In this medium, the bulk of the alkaloids is excreted into the medium. In studies of secondary metabolites of *Aspergillus* fungi, the latter are grown in surface culture on rich media. In this study, we used synthetic Abe medium to investigate the capacity of *Aspergillus* for producing indole alkaloids. During the analysis of secondary metabolites in the cultures under study, we focused on three major groups of indole alkaloids: clavines, α-cyclopiazonic acid, and diketopiperazine alkaloids. Alkaloids were detected with Ehrlich reagent, which gives color reactions with indole compounds.

We found clavine alkaloids only in *A. fumigatus* (isolates S48, Al141, F24, F85, F9-2, and T11). This group included only one metabolite. It was stained lilac with Ehrlich reagent, which is typical of indole compounds with no substituents at C2 in the indole ring sys-



Indole alkaloids found in soil strains of *Aspergillus* fungi: (*1*) α-cyclopiazonic acid; (*2*) fumigaclavine B; (*3*) ditryptophenaline; (*4*) brevianamide F; (*5*) verruculogen; (*6*) fumitremorgin C; (*7*) metabolite 3; (*8*) metabolite 4.

tem. Its chromatographic mobility depended strongly on eluent alkalinity (R_f 0.16 in system I and R_f 0.39 in system II). The UV spectrum of the compound contained three bands typical of an indole chromophore, with λ_{max} 272, 281, and 290 nm. The mass spectrum contained the following set of fragments: M^+256(100), 239(58), 213(10), 197(15), 154(31), and 144(16), which is typical of indole alkaloids. This spectrum corresponds to two compounds: fumigaclavine and chanoclavine I, well resolved according to their chromatographic mobility. We identified the compound as fumigaclavine B by the chromatography of its mixture with a reference sample formerly isolated by us from *P. palitans* (figure).

Although agroclavine and elymoclavine were shown to be produced by *A. flavus* and *A. fumigatus* in early studies of alkaloids of microscopic fungi, we found none of them in isolates of these *Aspergillus* species [5]. Neither did we find notable amounts of fumigaclavine A, characteristic of *A. fumigatus* [5].

Thus, in contrast to fungi of the genera *Claviceps* and *Penicillium*, where clavine alkaloids are very common, only fumigaclavine B was detected by us in seven representatives of *A. fumigatus* of all the *Aspergillus* strains studied.

α-Cyclopiazonic acid (α-CPA) is of special interest due to its toxicity and wide occurrence in fungi of the genera *Aspergillus* and *Penicillium* [3, 4, 8]. In some species, most isolates are capable of α-CPA production. For example, Horn and Dorner studied over 1000 *A. flavus* isolates from soils of various regions of the United States and found α-CPA in 83% [12]. This compound was also found in the following *Aspergillus* species: *A. parasiticus, A. versicolor,* and *A. oryzae* [1].

Table 1. Occurrence of secondary metabolites in *A. fumigatus* isolates

Locality of soil sampling	Isolate no.	Brevianamide F	Fumitremorgin C	Fumigaclavine B	α-CPA	Verruculogen
Alupka	Al141	+	+	+		
	Al25-1	+	+	+		
Simeiz	S48	+	+	+	+	
	S33	+	+			
Tver	T11	+	+	+		
	T3	+	+		+	+
Foros	F24	+	+	+	+	
	F9-2	+	+	+		
	F85	+	+	+	+	
Pushchino	B48	+	+		+	+
	B17	+	+		+	+
	B44	+	+		+	+
	As1	+	+		+	+

Of all the strains studied, *A. phoenicus* As18 showed the highest rate of α-CPA production. The metabolite isolated from this fungus was stained lilac with Ehrlich reagent and had a UV spectrum characteristic of α-CPA, with maxima at λ 224, 254, 274 (shoulder), 282, and 292 nm. Its chromatographic mobility was identical to that of the sample formerly isolated from *P. palitans*. Mass spectrum: M^+336(77), 196(54), 182(100), 181(55), 167(12), 155(42), 154(60), 127(15), and 70(48).

α-Cyclopiazonic acid was found in the following strains: S33, F9-2, Al13-1, T11 (*A. fumigatus*); Al13-1-2, Al83, Al58, WSS1, Kd1, Kg2-1, As11, Rs12, To6, F65, F24 (*A. flavus*); A1-3 (*A. versicolor*); As15, As18, As23, B51, To1, S57, F39, F71, F31-1-1 (*A. phoenicis*); and To8 (*A. clavatus*). Note that it had not been found in *A. fumigatus*, *A. clavatus*, or *A. phoenicis* before. Thus, this alkaloid occurs with the same frequency in soil strains of both northern and southern areas of Russia.

Indole-containing diketopiperazine alkaloids widely occur in fungi of the genus *Aspergillus* [13]. We found metabolites of this class in isolates of four species: *A. flavus*, *A. fumigatus*, *A. clavatus*, and *A. ochraceus*.

Metabolite 1, stained lilac with Ehrlich reagent, isolated from strain was As11 (*A. flavus*). Its UV spectrum had absorption maxima characteristic of the indole chromophore. Its mass spectrum had an intense peak of the molecular ion with *m*/*z* 347, an intense fragment at *m*/*z* 130, and fragments with *m*/*z* 103 and 77, characteristic of 3-alkylindoles. The presence of the ion with *m*/*z* 91 was indicative of a benzyl moiety. This suggests that metabolite 1 is N-methyl-phenylalanyl-tryptophanyl-diketopiperazine, identical to that previously found in *A. flavus* [9].

Metabolite 2, isolated from strain S14 (*A. flavus*), had a high chromatographic mobility (R_f 0.72 in system II). Ehrlich reagent first stained it intensely yellow. With time or upon warming, this color shaded to dark golden. The UV spectrum of the metabolite had absorption maxima with λ_{max} at 244 and 303 nm. The mass spectrum of the compound showed an intense molecular-ion peak and peaks for a fragment corresponding to a half of it, benzyl cation, and fragments characteristic of indole conjugated with the diketopiperazine ring: M^+692(100), 347(57), 346(68), 318(6), 256(12), 255(13), 157(32), 130(55), and 91(19).

Formerly, a compound with such properties was isolated by Springer *et al.* as an *A. flavus* metabolite and identified as ditryptophenaline, which is a dimer of N-methyl-phenylalanyl-tryptophanyl-diketopiperazine [14]. To confirm the structure of metabolite 2 as ditryptophenaline, it was hydrolyzed with diluted acetic acid according to [15]. As expected, the physicochemical properties of the hydrolysate matched those of metabolite 1. (N-methyl-phenylalanyl-tryptophanyl-diketopip-

Table 2. Physicochemical properties of the diketopiperazine alkaloids isolated from *A. fumigatus* isolates

Metabolites	Staining with Ehrlich reagent	Chromatographic mobility ($R_f \times 100$) in systems		UV spectra, λ_{max}, nm	Major characteristic peaks in mass spectra, *m*/*z*, %
		II	IV		
Brevianamide F	Lilac	39	35	222, 272, 281, 289	M^+283(7), 154(4), 130(100), 103(8), 77(9), 70(10)
Metabolites 3 and 4*	Lilac	65	17**	224, 274, 282, 290	M^+351(10), M^+349(18), 294(6), 251(15), 198(100), 182(23), 169(10), 168(10), 167(11), 130(9), 69(11)
Fumitremorgin C	Green	74	65	224, 272, 292	M^+379(100), 364(18), 336(10), 324(37), 282(41), 281(97), 227(34), 212(65), 199(26)
Verruculogen	Hazel	81	72	225, 275, 294	M^+511(100), 479(27), 429(10), 356(13), 338(29), 311(43), 259(32), 241(26), 214(28), 201(41), 174(17), 158(17), 130(8), 70(50)

* Mixture of two compounds.

** In system III.

erazine.) Ditryptophenaline was found in 14 out of 19 *A. flavus* isolates investigated by us, and N-methyl-phenylalanyl-tryptophanyl-diketopiperazine, only in 5.

The isolates of the most toxic species studied by us (*A. fumigatus*) produced, in addition to α-CPA and fumigaclavine B, five indole-containing diketopiperazine alkaloids (Table 1). We identified them as typical metabolites of *A. fumigatus*: prolyl-tryptophanyl-diketopiperazine (brevianamide F), fumitremorgin C, and verruculogen [4, 13, 16]. Metabolites 3 and 4 were present in minor quantities, and we could not resolve them by TLC. Their structure includes brevianamide F containing a dimethylallyl moiety. According to mass spectrometry, the molecular weights of these two compounds differ by 2 Da (Table 2). Insignificant differences in the intensity of their molecular ions (349/351) were observed during evaporation of the samples in the ionization chamber, but we failed to obtain individual spectra of these compounds by heat vacuum distillation because of the identity of their main fragment ions. The maximum intensity of the ion with *m*/*z* 198 is indicative of the presence of a dimethylallyl moiety at C_2 of the indole ring system and the absence of a second bond with the diketopiperazine ring. Physicochemical evidence suggests that the compounds are either prolyl-2(1',1'-dimethylallyl)tryptophanyl-diketopiperazine and its 12,13-dehydro derivative, previously found in *A. ustus* [17], or tryptostatin B and its dehydro derivative [16]. These metabolite pairs differ in the isomerism of the dimethylallyl moiety at C_2 of the indole ring system. Brevianamide F is a precursor of fumitremorgin C and verruculogen (figure). The physicochemical properties of these compounds are shown in Table 2.

We also detected brevianamide F in four out of nine *A. clavatus* strains (S20, Al25-1, Al50-3, and F36-1). The metabolite was isolated from the most active producer, S20, by preparative TLC in system I and purified in system III. Its physicochemical properties corresponded to those reported in [18] and the properties of similar metabolites found in *A. fumigatus* and *A. clavatus*. This is the first finding of brevianamide F in *A. ochraceous*.

The sole isolate of *A. clavatus* also produced brevianamide F, in addition to α-CPA. No indole-containing metabolites were found in the soil strains of *A. ustus* or *A. niger* isolated by us.

Thus, most isolates of *A. flavus* and *A. fumigatus* produce indole alkaloids. The clavine alkaloid fumigaclavine B was found in metabolites of a single species, *A. fumigatus* (46% of isolates of this species). α-Cyclopiazonic acid occurred widely in various species of the genus *Aspergillus*: 26.5% of the total number of isolates. Indole-containing diketopiperazine alkaloids were found in isolates of the species *A. flavus*, *A. fumigatus*, *A. clavatus*, and *A. ochraceus*. Brevianamide F was for the first time isolated from isolates of *A. clavatus* and *A. ochraceus*.

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