

Low Molecular Metabolites Of Fungi. Dimetoxystachibotrin From Stacybotrys Chartarum

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ABSTRACT

As a result of studies carried out in our country, information was obtained on the distribution of fungi of the genus *Stachybotrys*, their morphology, ultrastructure, cytology, biotechnology of cultivation on cellulose-containing nutrient media, ensiling of such solid plant waste as guzapoya, rice straw, corn stalks.

Stachybotrys chartarum produces a number of low molecular weight compounds. A technique has been developed for the isolation and separation of the sum of extractive substances - waste products of the fungus *Stachybotrys chartarum* grown under laboratory conditions on various nutrient media.

Cause and formulation of the problem, purpose of the work: The purpose of the study is the selection of active local strains of micromycetes, forming dimetoxystachibotrin and stachibotral, the study of their morphological cultural properties, the creation of a complex biological product based on promising local strains that protect against fungal phytopathogens.

Results / conclusions: Studied 3 strains of fungi of the genus *Stachybotrys* isolated from the rhizosphere of agricultural crops of serozem soils of Tashkent region and available in the collection of the laboratory.

As a result of studying the cultural morphological features, the identification of the strains of *Stachybotrys chartarum* was carried out and it was recommended to cultivate in a nutrient medium supplemented with 2% sucrose and 2% molasses.

As a result of screening the selected microorganisms, new local strains of *Stachybotrys chartarum* were obtained and identified with antimicrobial activity, antagonistic properties against phytopathogens and forming a wide range of 13,24-dimetoxystachibotrin and stachibotral in a liquid culture medium.

The optimal nutrient medium was selected for the selected strains (the influence of various carbon sources) and the cultivation conditions for the production of the greatest amount of dimetoxystachibotrin and stachibotral under submerged conditions.

Keywords:

Stachybotrys chartarum, *Stachybotrys chartarum*, chromatography, spectrum, correlation, interactions, system, coordinate, mycoses, fungus, low molecular weight, metabolites.

INTRODUCTION. The use of hay and straw infected with the fungus *Stachybotrys chartarum* for feeding horses in the 1930s caused massive illness and great death of horses

(stachybotriotoxicosis). The latest period in the development of medical mycology, which began in 1951, is characterized by in-depth biochemical studies of pathogenic fungi, their

enzymatic complexes, as well as the search for chemically pure waste products of pathogenic fungi useful for specific diagnostics and therapy. As a result of the search for means and methods of chemotherapy for fungal diseases, which are widely carried out in many countries, a number of active drugs, nystatin, amphotericin, candidin, and other fungal natures, have been discovered.

Chemical study of toxic components (metabolites), including alkaloids of macro and microscopic fungi, revealing the nature of the active toxic principle. Causing mass poisoning of humans and animals, the development of methods for the isolation of individual components, the establishment of the chemical structure and modification of these substances. In order to neutralize the latter and determine the possible mechanisms of action on a living organism is an extremely urgent problem [1].

MATERIALS AND METHODS. In the course of the study of toxic metabolites of the genus *Stachybotrys* from rice crops *S. chartarum* ATCC 62765, *S. chartarum* ATCC 62765, six new phenylspirodrimans were isolated: stachibotrilactone acetate, 2a-hydroxystachibotrilactone, 2a-acetoxystachibotrilactone stachybotrilactone acetate, stachybotrilactachrylactam *chartarum* MRC 1422, *S. chartarum* Egypt 1 and *S. complementi* ATCC 20511 along with three known stachibotridials, stachibotramide and stachibotrilactone [2].

Screening for pancreatic cholesterol esterase (PCEase) inhibitors from *Stachybotrys* sp. F1839 (from a soil sample collected in Shizuoka Prefecture, Japan), eight new phenylspirodrimans F1839-A-F, F1839-I, F1839-J were isolated, as well as two known K-76 and stachybotrydial [3-6].

During the screening of ET antagonists from culture broths of microorganisms, three new dimeric phenylspirodriman stachybocins A, B and C from the fermentation broth of *Stachybotrys* sp. M6222 (from soil collected in Yamanashi Prefecture, Japan) [7-10].

In a study of the chemical diversity of marine microorganisms, *S. chartarum* was isolated from the tissue of the sea sponge *Niphates recondite* (from a coral reef collected in

Beibuwan Bay, Weizhou Island). 16 new phenylspirodrimans, called chartarlactams A-P, were obtained from the fermentation of *S. chartarum* rice along with eight known analogues of stachibotramide, 2-acetoxystachibotrilactamacetate, stachibotrilactam, stachibotrilactamacetate, F1839-A, F1839-D -benzenepropanoic acid) stachibotrilactam [11,12].

Research methodology. The nutrient medium was selected and the cultivation conditions were optimized for the *Stachybotrys chartarum* strain. It was based on Mandels' medium, which is considered optimal for growing mushrooms of the genus *Stachybotrys*. In order to reduce the cost of the nutrient medium, peptone and urea were excluded from the Mandels medium. Cellulose-containing substrates were used as the sole carbon source instead of 2% sucrose. Instead of $(\text{NH}_4)_2\text{HPO}_4$, $(\text{NH}_4)_2\text{SO}_4$ was recommended as a nitrogen source, which had a positive effect on low molecular weight metabolites.

The optimal time of cultivation of the selected strain for obtaining low molecular weight metabolites, as well as the maximum amount of 13,24-dimethoxystachibotrin, formed in the culture liquid, was determined. The study was carried out in liquid and solid nutrient media with the addition of wheat bran for 10 days in the dynamics of growth. It was found that when growing the *Stachybotrys chartarum* strain, the pH of the medium increases to the alkaline side, the greatest activity of enzymes occurs, mainly, on the 3rd day with deep cultivation and on the 2nd day with solid-phase cultivation. The largest amount of 13,24-dimethoxystachibotrin was formed on the 4th day at deep and 2-day at solid-phase cultivation.

Extraction. *Stachybotrys chartarum* grown on Mandels medium was extracted in two ways. In the first method, the mycelium was separated from the aqueous phase by filtration, dried, crushed, and the extractives were extracted with diethylether. The aqueous portion was treated with diethyl ether, concentrated, and the residue was combined with a methanol extract, since they are chromatographically homogeneous. In the second method, the mycelium separated from the aqueous phase

without drying was treated with ether while heating under reflux; in this method, the yield of extractives is higher. An additional extract was obtained from the aqueous portion by treatment with chloroform, which was added to the ether extract. In qualitative terms, the ethereal extract obtained by the second method is cleaner than methanol and better separated. The mycelium of the fungus *Stachybotrys chartarum* grown on Mandels' nutrient solution (3L) for 14 days was separated by filtration from the aqueous part.

Process. The filtered mycelium was placed in a flask with 60 ml of ether and heated in a water bath at 40-45°C. The ether was decanted, the operation was repeated three times. The combined ether extracts were concentrated, dried under vacuum. Residue 5.50 g. The aqueous portion was treated with chloroform, the latter was distilled and dried. Yield 0.103 g. Total weight of the extract (5.603 g).

Thin layer chromatography. Thin layer chromatography (TLC) was performed on Silufol plates. The substances were detected on TLC by spraying with a 25% ethanol solution of phosphoric tungstic acid followed by heating for 5 min at 100-110 °C.

For column chromatography, silica gels of the Silpearl and L brands, particle size 50-100 µm, were used. Silpearl was used to separate the metabolites of *Stachybotrys chartarum*. Purification and separation of the products of chemical transformations were carried out on columns with silica gel of grade L. The following solvent systems were used: 1) benzene-methanol (9: 1); 2) chloroform-methanol (9: 1); 3) chloroform-methanol (8: 2); 4) chloroform-methanol (1: 1).

Mass spectra and elemental compositions of ions were measured on an MX-1310 instrument at an ionizing voltage of 50 eV and a temperature of 100 °C.

IR spectra were recorded on UR-20 and Perkin Elmer System 2000 FT-IR spectrophotometers in KBr.

^1H , ^{13}C , 2M ^1H - ^1H , ^1H - ^{13}C NMR spectra, chemical shift correlations (COZY) were recorded on a Bruker AM 400 instrument. ^{13}C NMR spectra were obtained with complete decoupling of C-H interactions and J-modulin. 2M NMR spectra of

long-range ^1H - ^{13}C interactions (HMBC) and NOE measurements in a rotating coordinate system (ROESY) were recorded on a Bruker AC 300 instrument. The spectra were recorded in deuteropyridine, unless otherwise indicated. All spectra were recorded using standard Bruker programs. ^1H NMR spectra of compounds **I** were recorded in deuteropyridine and compound **II** in deuteriochloroform were obtained on a Tesla BS 567 A (0-HMDS) spectrometer.

OBTAINED RESULTS AND THEIR DISCUSSION. The formation of 13,24-dimethoxystachibotrin and stachibotral in the growth dynamics by *Stachybotrys chartarum* strains and their antagonistic properties in relation to phytopathogenic microorganisms, the optimal days for the synthesis of 13,24-dimethoxystachibotrin and stachibotral by the selected strains were studied. The mushrooms were grown for 10 days in submerged conditions with the addition of sucrose and molasses to the nutrient medium in proportions from 1% + 1% to 5% + 5%. As a result, in all variants of nutrient media, the studied strains synthesized the greatest amount of dimethoxystachibotrin on days 6 and 7, and stachibotral on days 3, 6 and 9 of cultivation.

It was found that the *Stachybotrys chartarum* strain synthesizes a high amount of 13,24-dimethoxystachibotrin on the 6th day, respectively 1.167 mg / ml. It was noted that in the control variant, on the 6th day, the amount of 13,24-dimethoxystachibotrin was 0.749 mg / ml. (fig. 1)

It was also found that the greatest amount of stachibotral is synthesized on 3, 6, 9 days, respectively 0.271; 0.292 and 0.318 mg / ml. In the control variant, the same correlation was observed: on day 3, the amount of stachibotral was 0.076 mg / ml, on day 6, 0.087 mg / ml, on day 9 - 0.172 mg / ml. On the other day of the experiment, the number of stachibotral was significantly less.

The *Stachybotrys* sp. synthesized a larger amount of dimethoxystachibotrin on days 7 and 6, which amounted to 0.601 mg / ml and 0.635 mg / ml, respectively. In the control variant, the formation of 13,24-dimethoxystachibotrin on the 6th day was 0.475 mg / ml, on the 7th day it was 0.418 mg / ml. In both variants, an increase

in the amount of 13,24-dimethoxystachibotrin was observed on days 4 and 9.

Discussion. In the following experiments, an increase in the synthesis and formation of metabolites was found, associated with an increase in the concentration of sucrose and molasses. The highest amount of metabolite formation by all three studied strains was found in a nutrient medium with a concentration of 5% sucrose and 5% molasses, while the amount of dimethoxystachibotrin synthesized by the *Stachybotrys alternans* strain was 3 times higher compared to the control variant, and the amount of stachybotral 4 times. The amount of dimethoxystachibotrin synthesized by the *Stachybotrys* sp. there was 2.3 times more in comparison with the control variant, and the number of stachibotral was 3 times more. The formation of 13,24-dimethoxystachibotrin by the *Stachybotrys chartarum* strain showed 3.4 times more than the control variant, and the amount of stachibotral was 4 times higher.

It was found that the strains of the fungi *Stachybotrys alternans*, *Stachybotrys* sp. and *Stachybotrys chartarum* form the largest amounts of 13,24-dimethoxystachibotrin and stachybotral in a nutrient medium enriched with molasses.

During the research, it was found that the *Stachybotrys chartarum* strain is a producer of metabolites with high antibiotic activity against a number of micromycetes.

Isolation of dimethoxystachibotrin. Dry chloroform extract of *Stachybotrys chartarum* strains (25.8 g) was applied to a column containing 300 g of Silpearl silica gel. The column was eluted sequentially with benzene

and system 1. When the column was eluted with system I, 45 mg of dimethoxystachibotrin was isolated.

Dimethoxystachibotrin (I), $C_{27}H_{39}NO_5$, m.p. $211^{\circ}C$ (from MeOH), $R_f = 0.48$. (TLC, silufol, system 1), $[\alpha]_D^{24} = 14.5 \pm 2^{\circ}$ (benzene-methanol (9: 1)).

IR spectrum (KBr, ν , cm^{-1}): 3350-3140; 1675; 1650; 1630; 1475; 1360.

Mass spectrum, m/z (%): $M + 457$ (95) [457, 2483; $C_{27}H_{39}NO_5$], 439 (17.5) [439, 2401; $C_{27}H_{37}NO_4$], 412 (100), 394 (11.3), 367 (16.3) [367, 2319; $C_{23}H_{29}NO_3$], 274 (10), [274, 1097; $C_{15}H_{16}NO_4$], 260 (10), 256 (7.5), 242 (10), 234 (12.5), 223 (30), 221 (20), 189 (15), [189.1649; $C_{14}H_{21}$], 149 (12.5), 135 (12.5), 129 (12.5), 109 (12.5).

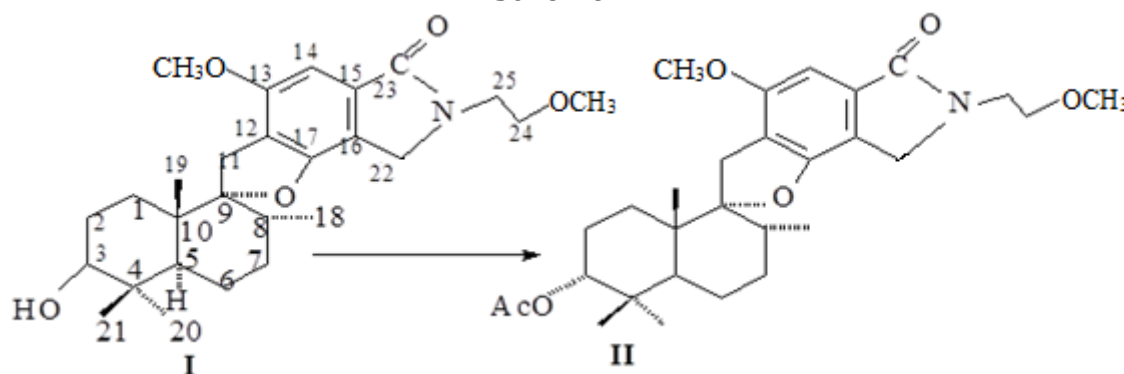
1H NMR spectrum - see table 1.

3 - 13,24-dimethoxystachibotrin (II) monoacetate from **I**. 13,24-dimethoxystachibotrin (14 mg) was acetylated with 0.5 ml of acetic anhydride in 1 ml of absolute pyridine at room temperature for 1 vessel. After evaporating the solvents, the residue was chromatographed on a column eluting with system 3. 10 mg of amorphous monoacetate **II**, $C_{29}H_{41}NO_6$, $R_f = 0.38$ (TLC, silufol, system 2) was isolated.

This article presents the results of establishing the structure of this compound.

Column chromatography of the sum of the waste products of the fungus *Stachybotrys chartarum* grown under laboratory conditions identified the dominant component in terms of content, which we named 22-methoxystichibotrin (Scheme 1).

Scheme 1



IR spectrum (KBr, ν , cm^{-1}): 1765; 1745; 1696; 1615; 1460; 1417; 1386; 1369.

Mass spectrum, m/z (%): $M + 499$ (50), 483 (25), 468 (100), 452 (9.2), 439 (13.1), 423 (5.3),

410 (7.8), 397 (6.9), 334 (6.9), 316 (2.6), 284 (5.3), 256 (19.7), 129 (35.7), 107 (15.7), 97 (36.8), 91 (18.4), 83 (27.6), 73 (42.1), 69 (65.8), 55 (100).

Stachibotral (III). $C_{23}H_{32}O_4$ m.p. 168 °C (from MeOH), $R_f = 0.45$. (TCX, silufol, syst. 1), $[\alpha]^{D_{24}} = 13.5 \pm 2^\circ$ ($CHCl_3$ -MeOH, 9: 1).

Mass spectrum, m/z $M + 372$ ($C_{23}H_{32}O_4$); 354; 339; 325; 216; 207; 189; 135.

1H NMR spectrum - see table 1.

Stachibotral (IV) 13-monoacetate. To 10 mg of a mixture of substances 3 in 1 ml of absolute pyridine was added 0.5 ml of acetic anhydride, and the reaction mixture was left at room temperature for 1 hour. The residue after evaporation of the solvents was chromatographed on a column, eluting with system 2. 9.5 mg of a mixture of monoacetates IV was isolated.

Table 1

NMR data of 1H , ^{13}C , compounds dimethoxystachibotrin (I) and stachibotral (III) (δ , ppm, C_5D_5N , 0-TMS)

Atom C	Connection			
	I		III	
	δ_c	$\delta_H(J, Hz)$	δ_c	$\delta_H(J, Hz)$
1	24.71	α 2.27 тд (13; 3,5) β 1.10 дт (13; 3,4)	24.80	
2	26,07	β 1,98 тт (13; 3,4)	26,09	
3	74,82	3,60	74,72	3,60 м
4	38,25	-	38,25	-
5	40,39	2,57 дд (13; 2,5)	40,50	2,60 дд (13; 2,7)
6	21,32	β 1,43 кд (13; 3,5)	21,38	
7	31,60		31,60	
8	37,32		37,34	
9	99,19		99,47	-
10	42,75		42,79	-
11	32,59	α 3,36 д (17) β 2,94 д (17)	32,80	α 3,40 д (16) β 3,00 д (16)
12	118,12		111,35	-
13	150,24		159,92	-
14	97,43	7,16с	111,86	6,52 с
15	135,75	-	129,20	-
16	115,32	-	111,71	-
17	156,72	-	141,86	-
18	15,87	0,78 д (6)	15,91	0,85 д (6,4)
19	16,19	0,99 с	16,27	0,97 с
20	29,15	1,22 с	29,20	1,24 с
21	22,74	0,91 с	22,72	0,90 с

22	48,48	4,11; 4,37 д (17)	21,85	2,68 c
23	168,85		168,92	10,87 c
24	60,54	3,98 м (2H)		
25	46,02	3,68; 3,98 м		
CH ₃ O-13	55,56	3,79 c		
CH ₃ O-24	52,35	3,52		

The chemical shifts given without multiplicities and coupling constants were determined from the ¹H-¹H COZY and HMQC spectra.

Conclusion / Application. The scientific significance of the research results lies in the fact that the formation of a wide spectrum of antibiotics, more precisely dimethoxystachibotrin and stachibotral, was noted in the culture liquid by selected local strains of the fungi *Stachybotrys chartarum*, the results obtained make a great contribution to the development of scientific research in our republic aimed at using environmentally fresh drugs.

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