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Abstract

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THE ACTIVE PRINCIPLE OF DENDRODOCHIN

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The study of the chemical nature of toxins of microscopic fungi that cause alimentary toxicoses in humans and farm animals is of great importance for elucidating the pathogenesis of the diseases and measures for combating them, as well as for deciding the possibility of the practical use of toxic substances in medicine and agriculture ([1,2]). Dendrodochin—a toxin formed in the course of the vital activity of the fungus *Dendrodochium toxicum* Pidopl. et Bilai—has sharply toxic properties for the animal organism. In acute experiments with laboratory animals, unpurified dendrodochin caused specific pathological-anatomical changes analogous to those observed in diseases of horses with dendrodochotoxicosis ([3–5]). Along with a generally toxic action on the animal organism, dendrodochin exerts, to a certain degree, a pronounced specific effect on the hematopoietic system, and also has a pronounced antifungal action against pathogenic and phytopathogenic species of mycelial fungi and yeasts.

At present it has been established that *Dendrodochium toxicum* has a fairly wide area of distribution. Toxin formation both in individual geographical races of the fungus and during cultivation has been insufficiently studied. Investigations carried out by N. M. Pidoplichko and V. I. Bilai showed that the biosynthesis of dendrodochin is a characteristic process in the metabolism of the fungus; the toxin was observed in the culture fluid and the mycelium of the fungus over a prolonged period of its growth. The activity of toxin formation depends on the strain under cultivation conditions ([6,7]).

Therefore it must be noted that the study of the chemical nature of dendrodochin, like that of other biologically active substances of microbiogenic origin, presents considerable difficulties. Extensive literature data indicate that, depending on the biological properties of the producers and the cultivation conditions, both compounds close in chemical nature (series) and very different ones may be formed in the course of metabolism. For the purpose of isolating the active principle and studying its chemical nature, we carried out experiments to select conditions giving the greatest yield of the unpurified preparation for its subsequent chemical study.

In the work, a strain of *Dendrodochium toxicum* was used that had been isolated in 1951 from soil of the Kherson region of the Ukrainian SSR and that, during the subsequent period of cultivation, had retained toxic properties for animals.

As a result of investigations conducted to study the physiological bases of dendrodochin biosynthesis, we used definite conditions for cultivating the fungus, taking into account the requirements for isolation and purification of the active principle.

Conditions for cultivating *Dendrodochium toxicum* for isolation of the toxin. Experiments on growing the fungus for the isolation of dendrodochin are carried out in fermenters of 24 l useful capacity with aeration of 1/2–1/3 volume of air per 1 min. Some experiments were conducted by cultivation in liter Erlenmeyer flasks with

800 ml of nutrient medium. In all, about 20 series of experiments were carried out; in each series the fungus was grown in two fermenters.

In connection with the task of studying the chemical nature of the active principle of dendrodochyin, the specific direction of the fungus' s metabolic processes is of great importance. Proceeding from this, for cultivation of *D. toxicum* in our experiments a mineral–carbohydrate medium of the following composition was used (in grams): sucrose 30, potassium nitrate 3, magnesium sulfate 0.5, potassium chloride 0.5, monobasic potassium phosphate 1 g, ferrous sulfate 0.01, water—about one liter. The medium may be prepared with distilled or tap water, in connection with which its initial pH usually ranges from 5.2 to 5.8.

Inoculation in the fermenters is carried out with a 48-hour inoculum grown on the above medium, in an amount of 5% of the volume of the culture liquid. The initial culture for obtaining the inoculum is grown on sterile bundles of a mixture of straw, bean, and cereal hay; its age is 10–30 days. On this medium the sporodochia of the fungus are formed abundantly, which provides the necessary quantity of seed spore material. To obtain the inoculum, flasks with 800 ml of nutrient medium are seeded with a suspension of conidia of *D. toxicum* from test tubes. The growth of the fungus in the inoculum consists of small uncolored, loose pellets; in the fermenters they are usually larger (up to 2–3 mm in diameter), dense, light flesh-colored or almost uncolored.

The duration of fermentation is 4 days. During the growth of the fungus, alkalinization of the culture liquid occurs, and by the end its pH reaches 6.8–7.3. During fermentation and at its end, samples are taken to determine sterility, pH, and certain other indices.

By the end of fermentation the culture liquid acquires a light yellowish-golden color and is transparent. It is separated from the mycelium through a single-layer cotton-paper (or nylon) filter. Filtration usually proceeds rapidly. After filtration is completed, a mean sample is taken for control.

After repeated washing with water on the same filter, the mycelium is collected, treated with acetone, and subjected to further study. Control of the experiments for determining the toxicity of the preparations obtained, as well as the course of fermentation, is carried out by the method of the skin test on rabbits (application of the toxin to depilated skin) and by antibiotic activity against a

microorganism selected by us—*Candida stellatoidea* 63.

Primary isolation of toxic substances from the culture liquid. The native culture liquid (pH 6.8–7.2) is acidified with 1 N HCl to pH 4.0–4.5 and extracted three times with chloroform. This achieves complete extraction of the toxic substances (the culture liquid extracted after 25-fold concentration is nontoxic). The chloroform extract is dried with sodium sulfate and evaporated at 30–35° in vacuo (water-jet pump) until the chloroform is completely removed. The residue is a viscous brown substance possessing high toxicity. The yield of the preparation, on average from 10 series of experiments, is 65 mg/l.

Purification of the crude preparation. For purification of the crude preparation, chromatography on Al_2O_3 (activity grade II) is used. For this purpose, 500 mg of crude dendrodochyin is dissolved in 10–15 ml of chloroform, and the resulting brown solution is chromatographed on a column 15–16 mm in diameter containing 30–35 g of Al_2O_3. After development with chloroform, a yellow-brown zone I was adsorbed in the upper part of the column; in the middle were colorless zones II, III, IV, and V, fluorescing under ultraviolet irradiation. After cutting the column into zones, the substance of each zone is eluted with an equal volume of ethyl alcohol (10 ml) and examined for biological activity (toxicity for animals and microorganisms). The substance of zone I could not be completely eluted; therefore it was tested in the form of the adsorbate.

Determination of toxicity showed the presence of maximum activity in zone IV; zones II and III are less toxic, while zones I and V are completely nontoxic.

Isolation of individual compounds. Further purification of the substances of each zone is carried out by repeated chromatography from chloroform on Al_2O_3.

Dendrodokhin I. After 3–5-fold chromatography of zone IV, a colorless crystalline substance possessing a high degree of activity was isolated (see Table 1). Further purification of the substance is carried out by recrystallization from a chloroform–petroleum ether mixture (1 : 4). The precipitating crystalline substance has m.p. 324° (decomp.). The substance obtained, which we named dendrodokhin I, is soluble in chloroform, benzene, and alcohol, poorly soluble in water, and insoluble in petroleum ether. The substance in alcoholic solution has a characteristic absorption maximum in the UV region, $\lambda = 250$ mμ, $\lg \varepsilon = 3.61$. The yield of dendrodokhin I is 1.0–1.2 mg per 1 l of culture fluid. Elemental analysis of the substance and determination of its molecular weight lead to the empirical formula $C_{17}H_{20}O_6$.

Table 1

Comparative toxicity of crude and crystalline dendrodokhin*

	Crude dendrodokhin	Crude dendrodokhin	Dendrodokhin I	Dendrodokhin I	Dendrodokhin II	Dendrodokhin II
	BS	BC	BS	BC	BS	BC
Minimal concentration in γ/ml inhibiting the growth of *Candida stellatoidea* 63	0.07	0.7	0.002	0.01	0.014	0.12
Minimal concentration in γ/ml producing a reaction on rabbit skin	12	12	0.01	0.01	0.15	0.15

* BS —bacteriostatic action; BC —bactericidal.

$C_{17}H_{20}O_6$. Found %: C 63.87; 63.77; 63.61; H 6.27; 6.18; 6.13
Calculated %: C 63.73; H 6.29

Determination of the molecular weight (cryoscopy in benzene) gave values in the range 320-340; calculated molecular weight for $C_{17}H_{20}O_6$ is 320.

Dendrodokhin II. Upon repeated chromatography of zone III, another crystalline substance with m.p. 217° (decomp.) was isolated and named dendrodokhin II. It also has a characteristic absorption maximum in the UV region, $\lambda = 262$ mμ; $\lg \varepsilon = 3.91$.

Determination of the elemental composition and molecular weight of dendrodokhin II led to the empirical formula $C_{14}H_{21}O_5$.

$C_{14}H_{21}O_5$.	Found %:	C 62.88; 62.61; 62.59;	H 7.71; 7.81; 7.79
	Calculated %:	C 62.43;	H 7.85

Determination of the molecular weight (cryoscopy in benzene) gave values in the range 265–294; calculated molecular weight 269.

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Note: Figure translations are in progress. See original paper for figures.