# METABOLITES OF THE PATHOGENIC FUNGUS *Verticillium dahliae*

## II. COMPONENTS OF THE FRACTION OF NEUTRAL LIPIDS FROM THE CULTURE LIQUID

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Results obtained in recent years leave no doubt of the fact that the lipid components of microorganisms largely predetermine the outcome of the interaction of a parasite and its plant host [1, 2]. In addition to the high biological activity of the oxidation products of lipids, an important role in the pathological process is played by the capacity of a mixture of lipids for transporting substances of nonlipid nature which, under certain conditions, may lead to the development of a chain process of the oxidation of the lipids in cell structures [2].

The present paper describes an aromatic diester and melanins forming components of the fraction of neutral lipids of the causative agent of verticilliaceous wilt of the cotton plant, Verticillium dahliae Kleb., which may be involved in the mechanism of the synergistic action of the metabolites responsible for the pathenogenicity of the fungus.

It has been shown by chromatography and nuclear magnetic resonance that the main components of the neutral lipid fractions of the culture liquid of the fungus V. dahliae are triglycerides of various degrees of unsaturation [3]. After separation on a column, from the combined substances agreeing in their $R_f$ values with the free fatty acids, we isolated a colorless liquid with a characteristic odor. The primary evaluation of the biological activity of the substance isolated showed its capacity for causing necrotic spots when applied to the surface of the leaf of a cotton plant.

In the proton magnetic resonance of a solution in carbon tetrachloride of the compound isolated (Fig. 1a), a multiplet at 7.5 ppm, corresponds to the protons of an ortho-substituted benzene ring [4]. Doublet splitting in a signal at 4.1 ppm shows the presence of a grouping of the type of $-O-CH_2-CH-$. Intense signals at 1.3 and 0.9 ppm are due to the protons of methylene and methyl groups, respectively. The carbon magnetic resonance spectrum with the suppression of the signals from the protons of the same solution contains 12 resonance peaks (Fig. 1b). The assignment of the signals on the basis of a spectrum with incomplete proton decoupling and the typical values of the $^{13}C$ chemical shifts [5-7] is as follows: 166.1 ppm – carbons of carboxy groups; 132.7 ppm – carbons of a benzene ring bearing equivalent substituents; 130 and 128.5 ppm – carbons of a benzene ring in the $\beta$ and $\alpha$ positions to a substituent; 67.2 ppm – carbons of $-OCH_2$ groups; 38.8 ppm – tertiary carbons; 30.4 and 29 ppm – carbons of a methylene chain in the $\gamma$ and $\beta$ positions to the terminal methyl group; and 23.8 and 22.9 ppm – carbons in the $\alpha$ position to methyl groups resonating at 14 and 11 ppm, respectively.

In order to harmonize this assignment of the $^{13}C$ signals with the results of the integration of the proton spectrum, it must be assumed that this compound is phthalic acid completely esterified with 2-ethylhexanol. Thus, on the basis of an analysis of the spectra given, the compound has been identified as di-2-ethylhexyl phthalate (I), which is known in the literature as a synthetic repellent and is used as a plasticizer for polymers [8]. The results of a comparison of its IR and mass spectrum with the spectra of synthetic (I) described in the literature [8] show their complete identity both in the aromatic and in the alkyl parts, which confirms the hypothesis that we made on the basis of the $^1H$ and $^{13}C$ NMR spectra.

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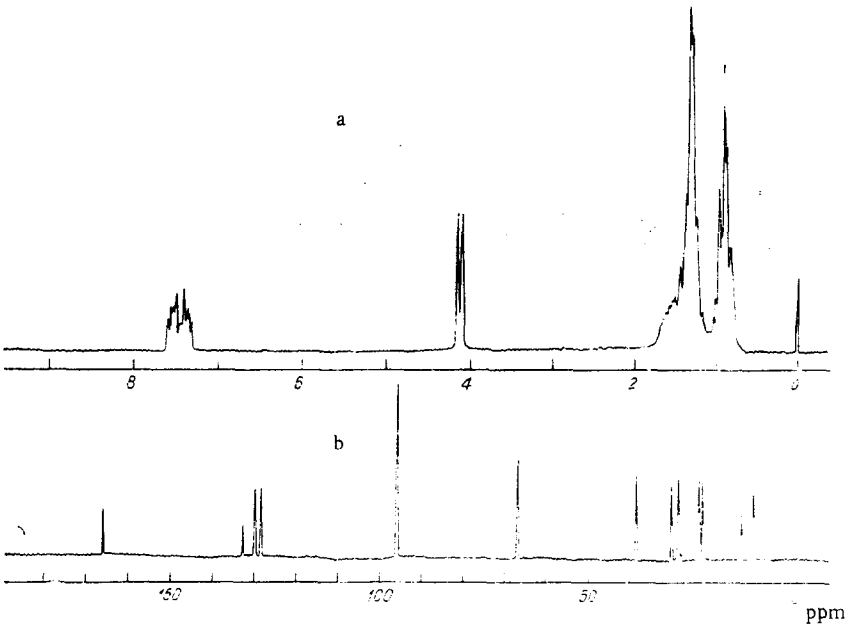


Fig. 1. $^{1}H$ (a) and $^{13}C$ (b) NMR spectra of compound (I) in $CCl_4$. The intense peak at 96 ppm is due to the nuclei of the solvent.

The toxicology of phthalic esters [including (I)] has been widely studied [8]. The capacity of exogenous and endogenous phthalic esters for accumulating in the cell lipids and inhibiting the growth of some species of rice is known [9]. The possibility of the biosynthesis of phthalic acid esters has been shown for one species of higher plants using labelled shikimic acid as precursor [10]. There is a report of the detection of (I) in the lipids of Hydrogenomonas eutropha [11], but the use by the authors of this paper [11] of labelled carbon dioxide to confirm the biosynthesis of (I) by this bacterium did not give an unambiguous conclusion. In our case, the considerable yield of (I) from the neutral lipid fraction enables us to regard it as a metabolite of the fungus V. dahliae.

In the separation of the mass of neutral lipids by column chromatography, seven chromogenic fractions were obtained, three of which possessed a well-defined capacity for causing the rapid appearance of the symptoms of verticilliaceous wilt when their aqueous solutions were injected into the atoms of sprouts of the cotton plant, both of varieties resistant to and of those unresistant to wilt. It has been established by electron spin resonance (ESR) that these pathogenic fractions differ from the nonpathogenic fractions by a high content of free radicals. The ERS spectra of these pathogenic fractions form slightly asymmetric singlet signals with a value of the g factor close to that characteristic for a free electron and a width at half height of about 6 Oe. Calculations show that the concentrations of paramagnetic particles in these fractions were, respectively, $1 \cdot 10^{19}$, $0.4 \cdot 10^{17}$, and $1 \cdot 10^{17}$ spins per gram of dry matter. These results agree to within an order of magnitude with those given in reports by other authors of the numbers of paramagnetic particles in some species of soil hyphomycetes [12].

The absence of a hyperfine structure from the ESR spectra is probably explained by the superposition of individual lines of different radicals of monomers present in the fractions investigated. The only slight difference of the value of the g factor of spectroscopic splitting obtained in our experiments from the g factor of a free electron is evidence of the closeness of the properties of the unpaired electrons responsible for the ESR absorption in these fractions and the properties of electrons in polymer structures. In the IR spectra of the pathenogenic fractions, broad bands were observed at 3 and 6 nm, which is also characteristic for polymeric structures. As is well known [13], compounds with a high content of unshared electrons per unit weight are, as a rule, insoluble in organic solvents, as is also found for these fractions. The facts given, and also the absence of characteristic peaks in the UV spectra and the uniform fall in absorption in the visible region with a slope of the straight line between $-0.002$ and $-0.04$, in combination with the presence of absorption bands at 3 and 6 nm in the IR spectra of the fractions studied were additional grounds [14] for assigning them to the class of melanina. Among the products of their alkaline hydrolysis on chromatographic analysis we found indole and pyrrole groupings.

The direct participation of the melanins of a microorganism in ensuring its parasitism – namely the pigments of the cytogenic fungus Venturia inaequalis – has been reported in the literature [15]. Attention is

merited by the fact that extracts from the culture liquid of the fungus V. dahliae obtained before the state of its pigmentation possessed no pathogenic action and showed no ESR absorption. The drying of the culture liquid in the stage of pigmentation, instead of the usual methods of preparing biological material for investigation by the ESR method, enabled the ESR signals that could have originated from other metabolic products to be eliminated. The ESR signal of the dried mass did not differ in shape and characteristics from the spectrum of the melanins isolated. The change in the concentration of free radicals with the development of the culture was in complete agreement with the level of accumulation of melanins, which may show the participation of these compounds in the development of the pathological process in the plant.

## EXPERIMENTAL

A Yangiyul' population of the fungus V. dahliae was grown in Czapek–Dox medium by the surface method in flasks in 28°C. To isolate the neutral lipid fractions, the culture liquid (500 liters) was separated from the 20-day mycelium by centrifuging followed by filtration through a double paper filter. The culture liquid (3 × 10 liters) was extracted with diethyl ether–petroleum ether (bp 40-60°) (1 :1 by volume). The extract was dried with sodium sulfate, filtered, and evaporated in a rotary evaporator, to give 6 g of an oil. Chromatography on columns (1:20) containing alumina ("chromatographic" grade) in the petroleum ether–hexane–methanol (10 :1 :1) solvent system led to the separation of the melanins fraction (0.3 g), which remained at the start, from the neutral lipids fraction (3.3 g). The subsequent separation of the neutral lipids was performed by thin-layer chromatography on fixed (gypsum) silica gel plates (type G, 20 × 20) using the hexane–ether (19 :1) and petroleum ether–diethyl ether–acetic acid (70 :30 :1) systems. On a thin-layer chromatogram, two spots were detected on the plate, which had been heated to 160°C, first with a 10% ethanolic solution of phosphomolybdic acid, and then with a 50% solution of sulfuric acid. Analysis of the substance corresponding in its $R_f$ value to the free acids showed that it was an aromatic diester with a molecular weight, according to mass spectrometry, of 390. Determination of its elementary composition gave the empirical formula $C_{24}H_{38}O_4$.

The melanin fraction (0.3 g) separated from a paper filter, was dissolved in water and deposited on a column (50 × 2) containing Molselekt G-15 at a rate of elution of 1 ml/sec. Seven chromogenic zones were eluted successively.

The proton and carbon magnetic resonance spectra were recorded on a Varian XL-100-15 multinuclear spectrometer at frequencies of 100 and 25.2 MHz respectively. The Fourier transformation of the $^{13}C$ signals was performed with the aid of a Data 620-i computer. As the standard for reckoning the chemical shifts in the $^1H$ spectrum we used hexamethyldisiloxane; in the $^{13}C$ spectrum the difference in the chemical shifts of the carbon tetrachloride used as solvent and a standard (tetramethylsilane) was taken as 96 ppm. The accuracy of the determination of the chemical shifts was ± 0.1 ppm.

The concentration of free radicals in the paramagnetic fractions were determined on a RÉ-1301 spectrometer using diphenylpicrylhydrazyl as standard.

## SUMMARY

Di-2-ethylhexyl phthalate has been isolated from the neutral lipids fraction of the culture liquid of the fungus Verticillium dahliae Kleb. Its $^1H$ and $^{13}C$ magnetic resonance spectra have been obtained, and an assignment of the signals has been made.

On the basis of their UV, IR, and ESR spectra, the chromogenic components of the neutral lipid fractions have been assigned to the melanins.

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# COMPOSITION OF THE PHOSPHOLIPIDS OF THE COTTON PLANT *Gossipium barbadense*

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In recent years, papers have appeared with increasing frequency on the fatty-acid composition and distribution of fatty-acid radicals in phospholipids of plant origin [1-12], the majority of them having been devoted to the study of the lecithins [5-9], and a smaller number to the cephalins [1] and to the phosphatidylinositols [10-12].

Papers on the fatty-acid composition of the lecithins of the cotton plant [7-9] have appeared, and one of them [9] reports the nature of the fatty acids in position 2. There is no such information for the phospholipids of the fine-fibered cotton plant of variety 5904-I in the literature.

We give the results of a determination of the fatty-acid composition of the oil, of the total phospholipids, of the phosphatidylcholines (PChs), of the phosphatidylethanolamines (PEs), and of the phosphatidylinositols (PIs) of the cotton plant *G. barbadense* (variety 5904-I, 1970 crop), and also their position-distribution in the molecules of the PChs, PEs and PIs. The oil and the total phosphatolipids were extracted as described previously. The individual groups of phospholipids were isolated by column chromatography on silica gel and were subfractionated in a thin layer of silica gel in system 1.

The results of a gas chromatographic (GLC) analysis of the fatty acids (Table 1) showed that the fatty-acid compositions of the oil of the total phospholipids are identical qualitatively and similar quantitatively. The individual phospholipids contain the same fatty acids, but their ratios are different, and in order of increasing saturation they form the sequence PChs, PEs, PIs. The amount of unsaturated fatty acids in the PIs is less than that in the PChs and PEs by 26.9-24.1%, respectively, through a decrease in the amount of oleic and linoleic acids, and the amounts of unsaturated acids are greater through an increase in the amounts of palmitic and stearic acids.

The position distribution of the fatty-acid radicals in the phospholipid molecules was determined by enzymatic hydrolysis. For this purpose we used phospholipase $A_2$ from the venom of *Vipera lebetina* obtusa (Azerbaidzhan kufi) [6]. The enzymatic hydrolysis of these phospholipids with 0.1 M tris buffer at pH 10.28 took place completely, but the times of hydrolysis were different and increased in the sequence PChs, PEs, PIs. The completeness of hydrolysis was checked in a thin layer of silica gel in system 1. GLC analysis of

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