METABOLITES OF THE PATHOGENIC FUNGUS *Verticillium dahliae*

V. 9,10-DICHLOROSTEARIC ACID – A MINOR COMPONENT OF THE LIPID FRACTION

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The present paper describes the results of a study of a halogen-containing compound isolated from the neutral fraction of the lipids of Verticillium dahliae Kleb. [1]. According to the results of neutron-activation analysis, the amount of chlorine in the total lipid fraction of V. dahliae was 0.012 g/g.

A mixture of the methyl esters (ME's) of the fatty acids obtained by the acid methanolysis of the total neutral lipids was separated with the aid of urea into four fractions. A precipitate giving a positive Beilstein test was isolated by fractional crystallization from acetone at −60°C from the fraction not forming a clathrate with urea. Thin-layer chromatography (TLC) on silica gel with the additions of $AgNO_3$ in system 1 showed that the precipitate contained components corresponding in chromatographic behavior to the ME's of a $C_{18:2}$ acid (R_f 0.54) and of a $C_{18:1}$ acid (R_f 0.67) and to an unknown component with R_f 0.83 (I).

The compound with R_f 0.83 was isolated by column chromatography (CC) on Al_2O_3, being eluted with diethyl ether, and it was freed from impurities by preparative TLC on Al_2O_3 in system 2 [2]. After re-chromatography, the substance (I) had R_f 0.74 in system 2 and 0.83 in system 1 and gave a positive Beilstein test.

The NMR spectrum of (I) showed a complex signal in the 3.9 ppm region relating to a −CHCl−CHCl− group similar in form to the signal at 4.08 ppm for the ME of 9,10-dibromostearic acid [2]. According to the mass spectrum, the molecular weight M^+ is 366/368/370, which corresponds to an empirical formula for (I) of $C_{19}H_{36}Cl_2O_2$.

The IR spectrum contains, in addition to the absorption bands that are usual for the spectra of ME's of saturated fatty acids, bands at 650 and 1320 cm^{-1} which are characteristic for the −CHCl−CHCl− group [4a, b]. On the basis of the identity of the IR, ^1H and ^{13}C NMR, and mass spectra of (I) and those of its synthetic analog, the structure of (I) has been determined as the ME of 9,10-dichlorostearic acid:

$$CH_3(CH_2)_7CHClCHCl(CH_2)_7COOCH_3 \quad (I)$$

In the ^{13}C NMR spectrum of (I) in CCl_4 solution at a frequency of 25.2 MHz (Fig. 1), 16 individual signals of ^{13}C nuclei are observed with the following chemical shifts (ppm relative to TMS): 172.1 (C-1), 64.9 (C-9, C-10), 50.7 (OMe), 33.8 (C-8, C-11), 33.6 (C-2), 31.8 (C-16), 29.6 (C-15), 29.3 (C-5), 29.2 (C-4), 29.0 (C-6), 28.8 (C-14), 28.7 (C-13), 26.7 (C-7, C-12), 24.7 (C-3) 22.6 (C-17), 14.2 (C-18).

In comparison with the corresponding values in the spectrum of the ME of 9,10-dibromostearic acid [3] in this case the signals of the C-9 and C-10 atoms are shifted downfield by 6.1 ppm, the signals of the C-8 and C-11 atoms upfield by 0.5 ppm, and the signals of the C-7 and C-12 atoms upfield by 1.1 ppm. In addition, the carbon atom of the carbonyl group has undergone an appreciable shift in the low-field direction (by 0.3 ppm). The values of the chemical shifts of the other signals do not differ from these in the spectrum of the ME of 9,10-dibromostearic acid within the limits of the accuracy of the measurements (0.1 ppm) [3]. In the region of high masses in the mass spectrum of I there are the peaks of ions with m/e: M^+ 366/368/370, 335/337/339

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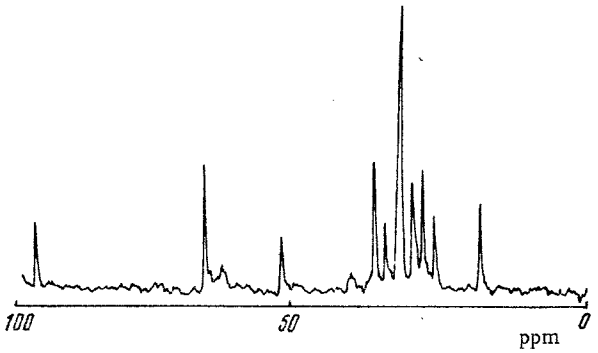

Fig. 1. ^{13}C NMR spectrum of a solution in CCl_4 of methyl 9,10-dichlorostearate.

($M-OCH_3$), 331/333 ($M-Cl$), 299/301 ($M-HCl-OCH_3$), 295 ($M-Cl-HCl$), 294 ($M-2HCl$), 264, 263. On the basis of the results obtained, it can be stated that the pathogenic fungus V. dahliae of the Yangiyul' population produces a chlorine-containing fatty acid, namely: 9,10-dichlorostearic acid (I).

When a fraction of the ME's of the fatty acids enriched with the ME of dichlorostearic acid was chromatographed by the TLC method on silica gel in systems 3 and 4, which are usually used for the separation of lipids into classes, secondary products of different polarities were observed. When the chromatograms were treated with 50% H_2SO_4 and were then heated to 130-150°C, some of these compounds were colored pink, similarly to the ME of dichlorostearic acid, and some of them lilac, similarly to di-2-ethylhexyl phthalate [5]. The PMR spectra of the compounds isolated (PTLC, silica gel) showed that the secondary products have an aromatic nature. Assuming that these products can be formed from the ME of 9,10-dichlorostearic acid in the process of separation, we studied the behavior of halogen-containing acids during their separation under the given conditions.

The total ME's of the acids of cottonseed oil with the addition of 5% of the ME of synthetic 9,10-dichlorostearic acid (sample A), 5% of the ME of 9,10-dibromostearic acid, and 5% of tetrabromostearic acid (sample B), and the ME of synthetic 9,10-dichlorostearic acid (80%: sample C) were separated by PTLC and CC on silica gel in system 3. In the products of the separation of sample A both by PTLC and by CC, in addition to the usual ME's we isolated a fraction of aromatic derivatives (R_f 0.96), esters of phthalic acid (R_f 0.76), and the ME's of methoxy (R_f 0.45) and hydroxy (R_f 0.28; system 3) derivatives of fatty acids. No methyl 9,10-dichlorostearate was detected in the fractions isolated (NMR). When sample B was chromatographed, fractions of aromatic derivatives, esters of phthalic acid, the ME's of the fatty acids of the cottonseed oil, all together with the ME of tetrabromostearic acid and with traces of the ME of dibromostearic acid, were isolated. The separation of sample C gave a main product in low yield (12%), a mixture of products of aromatic nature, and ester derivatives of phthalic acid.

On the basis of the results obtained, it may be considered that under the conditions used silica gel is capable of catalyzing the cyclization and polymerization of the ME's of halogen-containing fatty acids. It must be observed that when these samples were chromatographed in system 4 the amount and diversity of the secondary products was higher than with the other systems, which may be due to the presence of methyl ethyl ketone in the system [6]. One of the main secondary products was identified as di-2-ethylhexyl phthalate. We have previously isolated this compound from the extracellular lipids of a culture liquid of V. dahliae [5]. Its presence in the products of the vital activity of the fungus was confirmed by isolating the phthalate by methods excluding contact with silica gel (gel chromatography). The use of neutral Al_2O_3 as adsorbant for the isolation of halogen-containing fatty acids permits the formation of these secondary products to be excluded, but they are formed in very small amount when halogen-containing acids are chromatographed on alkaline Al_2O_3.

A chlorine-containing metabolite has been detected previously in the culture liquid of a fungus of the genus Fusarium [7]. We are the first to have found a chlorine-containing fatty acid in the lipids of V. dahliae. The presence of a chlorine-containing compound in V. dahliae indicates that it contains the active chloroperoxidase that has been detected in fungi and other microorganisms [8] where it performs halogenation.

EXPERIMENTAL

The conditions for recording the UV, IR, and ^1H and ^{13}C NMR spectra have been described previously [9, 10]. The mass spectra were recorded on a MAT-311 mass spectrometer. The fungus V. dahliae of the Yangiyul' population, strain L-1, was grown under stationary conditions in the dark at 26-28°C for 15 days. We have described the treatment of the mycelium, the isolation of the lipid fraction and of the fatty acids, and their partial composition previously [10]. The acid methanolysis of the triglyceride was performed by a method given by Christie [11].

Neutron-Activation Analysis. A sample under investigation with a volume of 0.5-2 ml was deposited on an ash-free filter paper with dimensions of 2 × 3 cm which was packed into a polyethylene bag and was then irradiated in the channel of a nuclear reactor. Samples and the appropriate standards were irradiated in a through vertical channel with a flux of $\sim 10^{13}$ neutrons/cm^2 · sec for 5-10 min. The induced activities of the sample and of the standards were measured by means of a semiconducting CTE (Li) detector in combination with a multichannel amplitude analyzer of type NTA-512B. The energy resolution of the spectrometric channel with respect to the γ line of ^{60}Co (1.33 Mev) was 4.5 keV. The chlorine was determined from the ^{38}Cl radioisotope ($T_{1/2}$ = 38 min, E_γ = 1.60 and 2.16 MeV) which is formed by reaction with thermal neutrons. The amount of chlorine was calculated from the clearly resolved 1.6 MeV photocurrent by a simplified method [15]. The sensitivity of the determination of chlorine for this type of solution is 0.012 g/g at a standard deviation of 10-15%.

The fractionation of the total ME's (20.6 g) with the aid of urea was carried out at a ratio of sample to urea to absolute methanol of 1:1:6. The mixture of reactants was heated until the urea had dissolved completely and was allowed to stand first at room temperature until it was cool and then in the refrigerator for 2 h. The further treatment of the precipitates and filtrates was similar to that described by Tvensan et al. [12].

The fractional crystallization of a 5% solution of the ME's in absolute acetone was performed with reduction of the temperature from −5°C to −70°C at intervals of five degrees.

Thin-layer chromatography (TLC) was carried out on L 5/40 silica gel (Chemapol) with the addition of 10% of gypsum and 3-5% of $AgNO_3$ (of the weight of the silica gel) in benzene (system 1); on Al_2O_3 (basic, Woelm) with the addition of 1% of gypsum in the petroleum ether (up to 50°C)−diethyl ether (9:1) system (system 2); and on silica gel with 1% of gypsum in the hexane−diethyl ether−98% acetic acid (70:30:1) system (system 3) and in the heptane−methyl ethyl ketone−acetic acid (41:9:0.5) system (system 4) [13].

In the case of TLC on Al_2O_3 the substances were revealed with a solution of I_2 in MeOH, and on silica gel with 50% H_2SO_4 followed by heating. The spots were identified by comparison with markers.

The total ME's (250 mg) were separated by CC on neutral Al_2O_3 (Reanal, Brockmann activity grade II), the dimensions of the column being 1.5 × 0.9 cm. Elution with diethyl ether (80 ml) yielded methyl dichlorostearate.

Methyl 9,10-dichlorostearate (I) (105 mg) formed a light yellow oil with M^+ 366/368/370. IR spectrum (film, cm^{-1}): 2960, 2870, 1740, 1440, 1370, 1320, 1255, 1200, 1180, 1100, 1025, 780, 650, 360, 630, ^1H NMR spectrum, ppm: 3.9 (2H, multiplet, CHCl−CHCl); 3.5 (3H, OCH_3, singlet); 2.15 (2H, CH_2-CO_2, triplet); 1.4-1.1 (26 H, $(CH_2)_{13}$, broadened singlet); and 0.8 (3H, CH_3, triplet).

The methyl esters of synthetic 9,10-dichlorostearic acid were obtained by chlorinating the total ME's of cottonseed oil [14] and separating the reaction products as described. The total ME's of the chlorine derivatives were separated by TLC on alumina in system 2.

SUMMARY

9,10-Dichlorostearic acid has been isolated from the lipid fraction of the mycelium of Verticillium dahliae Kleb. and its structure has been determined on the basis of IR, ^1H and ^{13}C NMR, and mass spectra, and also by comparison with the spectral characteristics of its synthetic analog.

It has been established that under the conditions of the methods of isolation and separation usually secondary products can be formed from halogen-containing acids present in a mixture of acids.

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METABOLITES OF THE PATHOGENIC FUNGUS Verticillium dahliae

VI. PENTAKETIDE METABOLITES AND NEUTRAL LIPIDS OF VIRULENT AND AVIRULENT STRAINS

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We have previously reported the isolation of phytotoxic metabolites and their action on isolated pea chloroplasts and on the permeability of synthetic phospholipid membranes [1-3] and also the composition of the extracellular (EL's) and intracellular (IL's) lipids of the fungus Verticillium dahliae Kleb. [4, 5]. In the present paper we give the results of a comparative study of the relative amounts of phytotoxic pigment from the culture liquid (PKZh-1) and the EL's and IL's in five strains and mutants of V. dahliae differing in virulence and in the nonpathogenic fungus V. lateritium when they are grown under stationary conditions.

We investigated the KhL-1,3 and KhL-1,7 strains of V. dahliae, the mutants R-196, R-101, and S-1, and V. lateritium [6]; for comparison we used information on the amounts of PkZh-1, EL's, and IL's in the wild Yangiyul' strain L-1: (see Table on following page)

As was found, PKZh-1 was present in the culture liquid of all the virulent strains, its amount being a maximum in KhL-1,3. This phytotoxic metabolite was not present in the nonpathogenic species, V. lateritium and the avirulent mutants, which shows a definite correlation between virulence and the amount of PKZh-1

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