METABOLITES OF THE PATHOGENIC FUNGUS *Verticillium dahliae*. I. ^{1}H and ^{13}C NMR SPECTRA OF THE EXTRACELLULAR LIPIDS

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UDG 537.635: 663.12

The chemistry of the metabolites of the causative agent of verticillium wilt of the cotton plant, *Verticillium dahliae* Kleb. unlike its mycology, has been little studied. This is due to features of the fungus culture itself and to the nature of the metabolites that are responsible for its pathogenicity. In the present communication we describe the results of a study by magnetic resonance methods of the extracellular metabolites of the fungus isolated from a 20-day culture liquid.

We have shown by the ESR [1] that the lyophilized mass of the culture liquid of the fungus *V. dahliae* contains a considerable amount of organic radicals which increases when microamounts of transition-metal ions are added to the culture during its growth. It has also been found that when the culture liquid is treated with ethyl acetate it is possible to separate from the residual mass of it an extract containing paramagnetic centers. It has been established that this ethyl acetate extract differs from other extracts of the liquid that have been tested by its capacity for causing well-defined chlorosis followed by necrosis of shoots of cotton plants of varieties 108-F and "Tashkent-1." This made it possible to assume that the pathogenicity of the culture liquid as a whole is connected with the components of this extract. In a further separation of the ethyl acetate extract we isolated oily fractions, the chromatographic characteristics of which permitted them to be considered compounds of similar structure. All the oily fractions isolated are insoluble in water, but they are readily soluble in organic solvents. Among the first three fractions obtained by the rechromatography of the total oily fraction the greatest yield (80% by volume) was represented by fraction I, the PMR spectrum of which is shown in Fig. 1, curve 1.

From the value of its chemical shift (CS), a triplet signal in the low field (5.2 ppm) can be assigned to olefinic protons. It was found by the double-resonance method that when this signal was suppressed by a strong radiofrequency field the doublet structure of the signal at 1.9 ppm disappeared. On the basis of this fact and the equality of the spin–spin coupling constants in these signals (5 Hz), the signal at 1.9 ppm corresponds to methylene protons in the α position to a double bond. A shift of the frequency of the strong radiofrequency field by 10 Hz in the direction of high fields, where the presence of a multiplet weaker than the signals of the olefinic protons (5.1 ppm in Fig. 1, curve 2) was assumed, led to the collapse of the doublet splittings with values of 5.5 and 4 Hz in the multiplet signal at 4.1 ppm. The 12-Hz spin–spin coupling constant in the multiplet at 4.1 ppm is characteristic for the spectrum of a type AB spin system [2] which, in combination with the results of double resonance, enables the multiplets at 5.1 and 4.1 ppm to be assigned to a type ABX system. The values of the CSs for the nuclei A (3.95 ppm), B (4.2 ppm), and X (5.1 ppm) make it possible to assume that they correspond to protons in the α position of oxygen atoms. In the integration of the spectrum, the signal of the AB part could serve as a natural calibration signal, for which it must be compared with the signal of the nucleus X, partially overlapped by the signal of the olefinic protons. A change of the solvent does not eliminate the superposition of the signals, but it shows that the CSs of the protons in the AB sys-

V. I. Lenin Tashkent State University. Translated from Khimiya Prirodnykh Soedinenii, No. 4, pp. 453-458, July-August, 1975. Original article submitted Febreruary 24, 1975.

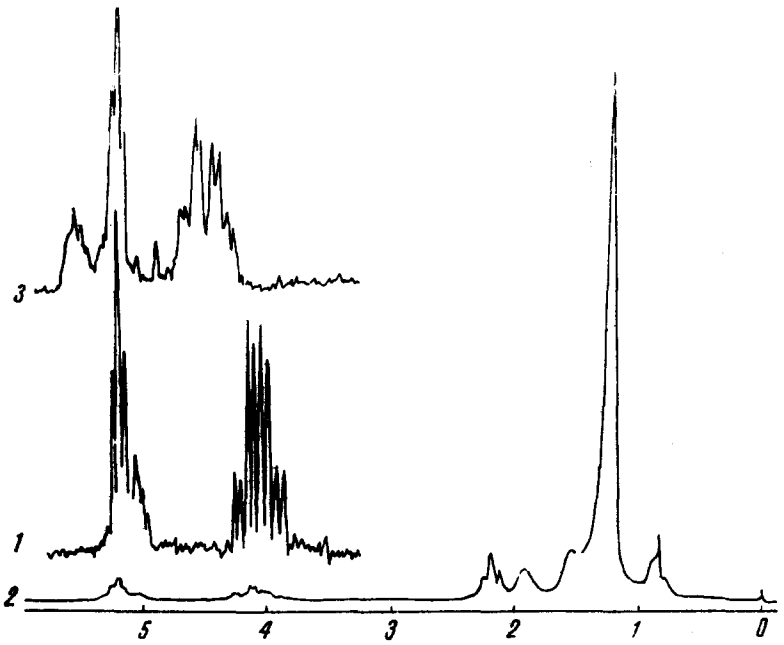

Fig. 1. PMR spectrum of a 20% solution of fraction I in CCl_4: 1) complete spectrum; 2) shape of the low-field signals at greater amplification of the apparatus; 3) shape of the same signals on the addition of a shift reagent to the solution.

TABLE 1. Parameters of the PMR Spectra of the Oily Fractions of the Culture Liquid of the Fungus *V. dahliae*

Chemical shifts in 10% CCl_4 solution (ppm) in fractions			J_H, Hz	Functional groups
I	II	III		
5,2	—	5,2	5	—CH=CH—
5,1	5,1	5,1	—	—OCH
4,2	4,2	4,2	12,4	$—OCH_2$
3,95	3,95	3,95	12, 5,5	$—OCH_2—$
—	—	2,7	—	$—CH_2—$ diene structure
2,2	2,2	2,2	7	$—OOC—CH_2—$
1,9	—	1,9	5	CH_2 in the α-pos. to a db.
1,2	1,2	1,2	—	CH_2 of a chain
0,8	0,8	0,8	—	CH_3 terminal

tem change. This also indicates the presence of two types of protons in the multiplet signal at 4.1 ppm.

To isolate the signal of the nucleus X we used the property of paramagnetic ions of the lanthanides of causing a shift of the signals of protons close to the coordination center of a ligand (effect of a shift reagent [3]). As was to be expected, the addition of small amounts (0.1 M) of europium trisdipivaloylmethanate to a solution of fraction I in CCl_4 caused a considerable shift of the signals of the protons closest to the oxygen atoms. In Fig. 1, curve 3, the signal of the X proton shifted downfield in relation to the signals of the olefinic protons can be clearly seen. The integration of the spectrum (Fig. 1, curve 3) gave a ratio of the areas of the signals of the X and AB parts of 1:4, which shows the presence of two AB systems with a common X proton. The results of a calculation of the spectrum of a A_2B_2X system which we performed by means of a "Spin Simulation" program [4] using the parameters given in Table 1 confirmed this conclusion. The molecular fragment the protons of which could correspond to the situation described could be a glycerol fragment. Thus, the values of the CSs and the multiplicities and intensities of the signals of the spin system under consideration confirm the presence of a glycerol fragment in fraction I. The results of a comparison of the spectrum obtained with the spectrum of a glycerol fragment in triacetin [5] confirmed that the signals at 5.1 and 4.1 ppm do actually belong to the protons of —OCH and $—OCH_2$, respectively. Qualitative reactions for a glycerol fragment [6] in fraction I were positive.

The intensive signal at 1.2 ppm (Fig. 1, curve 1) is due to the protons of the main chains of fatty acids. The terminal methyl groups are represented by a triplet signal in the higher field (0.8 ppm), and the signal of the methylene protons adjacent to the carboxy

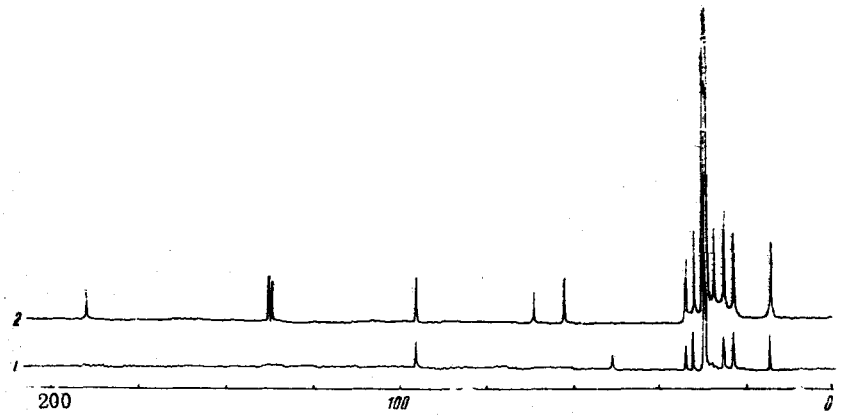

Fig. 2. Spectra of the ^{13}C nuclei with complete suppression of the protons in CCl_4 solutions: 1) fraction I; 2) methyl stearate.

group is shifted downfield (2.2 ppm). Since it is almost impossible to separate oils of natural origin into fractions of individual molecules [7], an accurate estimate of the number of methylene protons from the spectrum given is limited by the degree of homogeniety of fraction I achieved by rechromatography. Under these conditions the results of the integration of the signals mentioned permit us to consider that the ratio of the intensities of the signals of the olefinic protons to the others in order of decreasing CSs of the signals is 2:1: 4:6:4:80:10.

For a more complete identification of fraction I we recorded the magnetic resonance spectra of the carbon nuclei with the natural concentration of the ^{13}C isotope using pulse technique and Fourier transformation. Figure 2, curve 1, shows the ^{13}C spectrum of fraction I that we obtained under conditions of complete noise decoupling from the protons [8]. In the assignment of the signals we used literature information on the positions of the resonances of the carbon nuclei in various functional groups [9], and also the method of the strong nonresonance irradiation of the protons [10] to determine the number of hydrogen atoms attached directly to a particular carbon atom. In Fig. 2, curve 1, the signal in the low field at 171.4 ppm relates to the carbons of carboxy groups. The signal at 129.4 ppm belongs to the carbon atoms of a double bond which, under conditions of nonresonance irradiation of the protons, is confirmed by its doublet structure. The signals of the unsaturated carbon atoms are somewhat nonequivalent (Δ = 5 Hz), which is apparently due to the long-range influence of the carboxy group. The carbon atoms of the glycerol fragment are represented by peaks at 68.8 ppm (—OCH group) and 61.8 ppm ($-OCH_2$ group).

In the highest field (14.1 ppm) there is a signal showing a quartet structure under conditions of the nonresonance irradiation of the protons, which permits it to be assigned to the carbon nuclei of terminal methyl groups of fatty-acid residues. The remaining signals, showing a triplet structure on the nonresonance irradiation of the protons, are assigned to the nuclei of secondary carbon atoms. The positions of their peaks coincide with those in the ^{13}C NMR spectrum of oleic acid [11]. The strongest peaks at 29.6 and 29.2 ppm are due to the carbons of the main chains of the fatty-acid residues. Some difference in the relative intensities of these peaks and of the peaks of the carbon atoms of the double bond found on comparingthe spectrum that we obtained with the spectrum of oleic acid show the presence in fraction I of acids of the oleic series together with unsaturated and saturated residues.

Shifted downfield from the signals of the main chain (see Fig. 2) are the signals of the carbon nuclei in the α positions to the carboxy groups (33.7 ppm) and those in the β position to the terminal methyl groups (31.8 ppm). Shifted upfield from the signals of the main chain are the signals of the carbon atoms in the α positions to the double bonds (27.1 ppm), in the β positions to the carboxy groups (24.6 ppm), and in the α positions to the terminal methyl groups (22.6 ppm). The upfield shift of the signals of the nuclei of the carbons of the carboxy groups in fraction I by 9.1 ppm as compared with the corresponding signals in oleic acid shows that in this fraction all three positions of the glycerol fragment are occupied by fatty-acid residues. This is confirmed by the results of a comparison of the ^{13}C NMR spectrum of pure glycerol that we obtained with the spectrum of fraction I. Thus, the combination of the results of ^{1}H and ^{13}C NMR makes it possible to state that fraction I contains triglycerides with fatty-acid residues of the oleic series.

The relatively high yield of fraction I enabled us to accumulate it in an amount sufficient for saponification with subsequent analysis by Coleman's method [12]. Figure 2, curve 2, gives the ^{13}C spectrum of methyl stearate obtained from the products of the saponification of fraction I. Its comparison with the spectrum of fraction I itself (Fig. 2, curve 1) shows the correctness of the assignment of the signals made.

From the parameters of the PMR spectra of the other components of the total oily fraction, which are shown in Table 1, it follows that, in contrast to the main fraction, fraction II does not contain unsaturated fatty-acid residues. The presence of a signal at 2.7 ppm in the spectrum of fraction III shows that it contains unsaturated residues with dienic conjugation.

Thus, the fractions of the metabolites of the fungus *V. dahliae* that have been studied consist of a mixture of triglycerides with relative proportions of saturated and unsaturated fatty acids varying from fraction to fraction.

The presence of a dienic structure in the lipid fraction of the culture liquid shows that among the metabolites of the fungus there are compounds capable of converting the unsaturated fatty acid residues into hydroperoxides [13] which unavoidably arise as intermediate products in the formation of dienic structures by a free-radical mechanism. Consequently, the increase in the number of organic radicals in the culture liquid under the action of transition-metal ions observed previously can be explained by a catalytic acceleration of the oxidation of the components of the culture liquid, including the triglycerides described.

EXPERIMENTAL METHOD

An ethyl acetate extract was obtained by treating a 20-day culture liquid of the fungus *V. dahliae* of the Yangiyul' population grown on Czapek-Dox medium. The solvent was eliminated in a rotary evaporator, and the residue was treated with petroleum ether and separated into fractions by column chromatography. The oily fractions obtained were rechromatographed.

The ^{1}H and ^{13}C NMR spectra were recorded on a Varian XL-100-15 multinuclear spectrometer. The proton spectra were recorded at 100 MHz under continuous-flow conditions with a scanning time of 250 sec at a temperature of +38°C using HMDS as internal standard. The spectra of the protons of the glycerol fragment were calculated on a DATA 620 i computer. The ^{13}C NMR spectra were obtained at 25.2 MHz. The solutions of the samples investigated were placed in the inner part of a coaxial tube the outer part of which contained 2 ml of D_2O to ensure the stabiliztion of the resonance conditions. In the recording of the ^{13}C spectra we used the pulse method with accumulation and subsequent Fourier transformation of the free induction signal with the aid of the DATA 620 i computer. The accuracy of the determination of the chemical shifts was ±0.1 ppm. The europium trisdipivaloylmethanate was kindly given to us by Yu. A. Ustynyuk (Moscow State University).

SUMMARY

From the culture liquid of the fungus *V. dahliae* an extract has been isolated which contains paramagnetic centers and causes the symptoms of verticillium wilt of the cotton plant. By chromatography and ^{1}H and ^{13}C nuclear magnetic resonance it has been shown that this extract includes triglycerides and fatty-acid residues of the oleic series with various degrees of unsaturation.

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