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PHENOLIC LIPID FROM Azotobacter chroococcum

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In an investigation of the combined cell lipids of the nitrogen-fixing bacterium Azotobacter chroococcum 92 [1] by thin-layer chromatography (TLC) we found that one of its main components is an unusual comparatively feebly polar lipid giving the positive reaction with ferric chloride that is characteristic for phenols and enols. The present paper describes the isolation and structural identification of this substance (I).

The combined lipids of A. chroococcum 92 obtained by extracting the freeze-dried cells with mixtures of chloroform and methanol were subjected to ion-exchange chromatography on DEAE-cellulose [2]. The lipid (I) was eluted in the same fraction as the neutral glycerides with chloroform–methanol (9:1), which indicates the absence of strongly anionic groupings in its molecule.

The subsequent chromatography of this fraction on a column of silica gel led to the isolation of the lipid in the form of a crystalline substance which, on TLC in various solvent systems behaved as an individual lipid fraction. The lipid isolated was stable under the conditions of acid and alkaline methanolysis and did not change on treatment with sodium tetrahydroborate and lithium tetrahydroaluminate. Under the action of acetic anhydride in pyridine it gave an acetyl (II) or benzoyl (III) derivative, and under the action of diazomethane it was slowly converted into a methyl ether (IV). Analysis of the UV, IR, PMR, and mass spectra of the native lipid (I) and its derivatives (II-IV) permitted an unambiguous conclusion concerning its structure.

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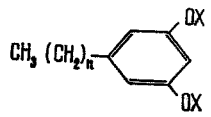


I.X=H, II.X=Ac, III.X=Bz, IV.X=Me; $n=17-22$

The IR spectra of the native lipid (I) contained a broad band of the stretching vibrations of H—O bonds of hydroxy groups with a maximum at about 3300 cm^{-1}, and no absorption due to carbonyl groups was observed. A number of bands (at 1629, 1602, 1512, 828, and 697 cm^{-1}) show the presence of an aromatic system in the molecule. This was confirmed by the UV spectrum of the lipid (I), which contained in the 220-300 nm region absorption maxima at 274 and 280 nm (1280 and 1240, respectively),* which are characteristic for a substituted benzene chromophore [3]. The IR spectrum of the acetyl derivative (II) showed no absorption of hydroxy groups but had a strong band at 1776 cm^{-1} which must obviously be assigned to the stretching vibrations of the carbonyl group of the phenol ester. In the IR spectrum of the benzoate (III) the band at 1742 cm^{-1} corresponded to the stretching vibrations of the same bonds. The facts given show that the molecule of the lipid (I) includes a benzene nucleus to which the hydroxy groups present are attached.

More detailed information on the structure of the lipid (I) was given by the PMR spectra of its acetyl (II) and O-methyl (IV) derivatives (Table 1).

The spectra of both derivatives have in the strong-field region a three-proton triplet at δ 0.89 ppm (J = 6.5 Hz) and a fairly narrow multiplet (~40 H) at δ 1.26 ppm, which shows the presence in the molecules of these compounds of an alkyl residue of normal structure with a long carbon chain. A six-proton singlet at δ 2.26 ppm in the spectrum of the acetate (II) must obviously be connected with the presence in the molecule of this derivative of equivalent acetoxy groups attached to an aromatic nucleus. Correspondingly, in the spectrum of the O-methyl derivative (IV) there is a six-proton singlet at δ 3.77 ppm from two methoxy substituents of a benzene ring.

The nature of the bond between the alkyl residue and the aromatic nucleus is shown by a two-proton multiplet with its center at δ 2.61 ppm in the spectrum of the acetate (II) or at δ 2.54 ppm in the spectrum of the methyl ether (IV). This signal has the structure of a triplet with J = 6.5 Hz, each element of which, in its turn, is split into three lines with J = 1 Hz.

The multiplet considered apparently corresponds to the protons of a methylene group of the "benzyl" type interacting both with the protons of a neighboring methylene group and with two equivalent aromatic protons present in the ortho position with respect to the "benzyl" methylene group. In other words, the aliphatic chain in the molecules of the derivatives (II) and (IV) and, consequently, also in the molecule of the lipid (I) must be attached directly to the benzene nucleus, and the two ortho C-atoms of the latter must be unsubstituted. Thus, the lipid (I) is a 1-alkyl-3,5-dihydroxybenzene. This conclusion is confirmed by an analysis of the signals from the aromatic protons. In the PMR spectrum of the acetate (II) these are represented by a one-proton triplet with J = 1 Hz at δ 6.74 ppm and a two-proton doublet with J = 1 Hz at δ 6.80 ppm; in the spectrum of the O-methyl derivative (IV) the analogous signals are located at δ 6.30 and 6.36 ppm. The facts given unambiguously indicate 1,3,5-substitution of the benzene nucleus.

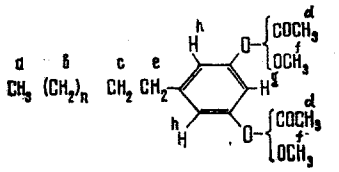
The definitive conclusion concerning the structure of the lipid (I) was made on the basis of the mass spectra of the derivatives (II), (III), and (IV) (Table 2). The spectra of all these derivatives contained strong peaks of homologous molecular ions the m/e values of which enable the size of the hydrocarbon chains of the alkyl residues to be determined. Thus, in the mass spectrum of the methyl ether (IV) the dominating molecular ions have m/e 432 and 404 and correspond to compounds with hydrocarbon chains of 21 and 19 carbon atoms.

The intensities of the peaks of these ions are in a ratio of 3 : 1, which obviously reflects the quantitative ratio of the corresponding homologs in the lipid fraction. The intensities of the peaks of the molecular ions of the other homologs (m/e 460, 446, 418, and 390) are very low, from which it may be concluded that the latter are present as minor components in the fraction.

Similar conclusion can be arrived at by analyzing the mass spectra of the acetyl (II) and benzoyl (III) derivative. It is true that in the case of the acyl derivatives (II) and (III) the molecular region of the mass spectrum consists of a more complex pattern because of the appearance of peaks of fragmentary ions arising as the result

*Here and below, in the calculation of ε the "mean" molecular weight of the lipid fraction found by mass spectrometry was used.

TABLE 1. PMR Spectra of the Acetyl (II) and Methyl (IV) Derivatives of Phenolic Lipid (I)

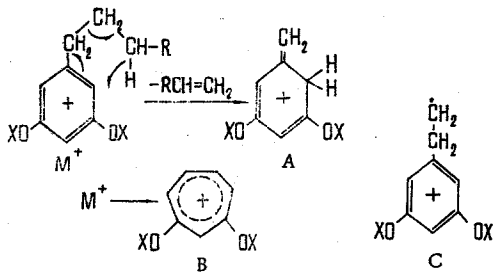


Proton	Number of H's	δ, ppm for the spectrum of (II)	δ, ppm for the spectrum of (IV)	Structure of the signal (J, Hz)
a	3	0,89	0,89	Triplet (6.5)
b		1,26	1,26	Multiplet
c	2	1,62	1,62	Multiplet
d	6	2,26	—	Singlet
e	2	2,61	2,54	Multiplet (6.5 and 1)
f	6	—	3,77	Singlet
g	1	6,74	6,30	Triplet (1)
h	2	6,80	6,36	Doublet (1)

TABLE 2. Main Peaks in the Mass Spectra of the Acetyl (II), Benzoyl (III), and Methyl (IV) Derivatives of the Phenolic Lipid (I) (the m/e values of the ions are shown, with the relative intensities, %, in parentheses)

Type of ion	Derivative		
	II	III	IV
M_1^+	488 (2,3)	612 (41,2)	432 (39,5)
M_2^+	460 (1,1)	584 (14,2)	404 (13,2)
$[M_1-X+H]^+$	446 (37,5)	508 (2,8)	—
$[M_2-X+H]^+$	418 (12,9)	480 (1,0)	—
$[M_1-2\cdot X+2\cdot H]^+$	404 (100)	—	—
$[M_2-2\cdot X+2\cdot H]^+$	376 (45,6)	—	—
C	—	—	165 (14,0)
A	208 (1,7)	332 (5,2)	152 (100)
B	207 (1,1)	331 (1,0)	151 (21,7)
$[A-X+H]^+$	166 (9,1)	228 (1,0)	—
$[B-X+H]^+$	165 (3,8)	—	—
$[C-2\cdot X+2\cdot H]^+$	137 (14,8)	—	—
$[A-2\cdot X+2\cdot H]^+$	124 (92,2)	124 (4,0)	—
$[B-2\cdot X-2\cdot H]^+$	123 (38,0)	123 (1,9)	—
X^+	43 (35,8)	105 (100)	—

of the elimination of the acyl substituents (in particular, in the mass spectrum of the acetate (II) the maximum peaks correspond to the fragment $[M - CH_2=C=O]^+$). In agreement with the structures proposed for the native lipid and its derivatives (I-IV), in the fragmentation under electron impact of the molecular ions the formation of two characteristic fragments was to be expected [4] – the ion A with an even value of m/e arising in the cleavage of the β-bond in relation to the aromatic nucleus with migration of a H atom to the charged fragment, and the substituted tropylium ion B, to which cleavage of the same bond but without migration of the hydrogen atom leads:



The peaks of the ions of both these types (or the products of the further fragmentation of these ions) have high intensities in the mass spectra of the derivatives (II-IV). For example, the peak of ion A is the maximum peak in the spectrum of the methyl ether (IV); in the mass spectra of the acyl derivatives (II) and (III) some of the strongest peaks are those of ions formed from the fragments of types A and B by the elimination of acyl residues with the migration of a H atom. It must be added that the peaks of fragments C arising as the result of the cleavage of the γ-bond of the aliphatic chain also have appreciable intensities in the mass spectra of the derivatives (II) and (IV). All these facts confirm the structures of compounds (I-IV) flowing from the characteristics of the PMR spectra.

Lipid derivatives of diphenols of chromanol, chromenol, and hydroquinone structures the hydrocarbon chains of which are made up of isoprene units (vitamins C and K, ubiquinones) are widespread in nature. In the cell they fulfil the functions of electron carriers in redox processes. Phenolic lipids of a normal hydrocarbon chain are a fairly rare phenomenon.

Apart from the strain A. chroococcum that we have investigated only one microorganism producing a lipid of a similar type has been described – the fungus Neurospora crassa [5]. The phenolic lipid fraction isolated from the cells of this organism consisted of a mixture of 6-n-heneicosyl-2,4-dimethoxyphenol and a derivative of it in the molecule of which the benzene nucleus was additionally substituted by a methyl group (the position of the latter was not established). It is known that the nitrogenases of nitrogen-fixing bacteria are extremely sensitive to atmospheric oxygen, and therefore they require suitable protection [6]. It may be assumed that the lipid (I) described in the present paper plays the role of one of such antioxidants.

EXPERIMENTAL

Materials and General Methods. The UV spectra were recorded on a Specord UV-Vis spectrograph (GDR) and the IR spectra on a UR-10 spectrograph (Zeiss, GDR) with the substances in the form of tablets with KBr. The PMR spectra were obtained on a XL-100 instrument (Varian, USA) at a working frequency of 100 MHz with tetramethylsilane as internal standard and CCl_4 as solvent. The mass spectra were measured on an LKB 9000 mass spectrometer (Sweden) at an energy of the ionizing electrons of 70 eV and an accelerating voltage of 3.5 kV; the samples studied were introduced directly into the ion source, the temperature of the evaporator being 80-90°C.

TLC was performed by the micromethod of Svetashev and Vaskovsky [7] on type KSK silica gel. The substances were revealed on the chromatograms by spraying them with 50% sulfuric acid followed by heating the plate at ~200°C, and also with a 0.1% solution of morin in MeOH followed by the observation of the spots of the substances in UV light.

The melting points of the substances (uncorrected) were determined on a "Boetius" heated stage (GDR).

For the investigation we used a culture of Azotobacter chroococcum 92 [1] bred in VNIIbakpreparat [All-Union Scientific-Research Institute of Bacterial Preparations] for antibiotic activity from an initial strain obtained from the collection of the Institute of Microbiology of the Academy of Sciences of the USSR. The culture was grown on Fedorov's nitrogen-free liquid synthetic medium in flasks with shaking on a circular shaking machine (200 rpm) at 30°C for 72 h. The cells were separated by centrifuging, washed with distilled water, and freeze-dried.

Extraction of the Total Cell Lipids. The freeze-dried cells (28 g) were stirred with 600 ml of a mixture of $CHCl_3$ and MeOH (1:1) at 20°C for 1.5 h. The undissolved matter was filtered off and was extracted with 600 ml of $CHCl_3$–MeOH (2:1) under the same conditions. The combined extract was evaporated to dryness in vacuum at a temperature < 30°C, the residue was dissolved in 150 ml $CHCl_3$–MeOH (2:1), the solution was shaken with 30 ml of water for 5 min, the lower phase was separated off and evaporated to dryness in vacuum, and the residue was dried at 25°C/0.02 mm for 3 h. This gave 2.6 g (9.3% of the total weight of the cells) of combined cell lipids.

Isolation of the Phenolic Lipid (I). A solution of 1.16 g of total cell lipids in 15 ml of $CHCl_3$–MeOH (9:1) was deposited on a column (2.5 × 30 cm) of DEAE-cellulose (Reanal, Hungary) in the AcO^- form [2]. The column was washed successively with the following solvent systems (300 ml each): $CHCl_3$–MeOH (9:1), $CHCl_3$–MeOH (7:3), $CHCl_3$–AcOH (4:1), MeOH, and $CHCl_3$–MeOH (2:1) + 10% of concentrated NH_4OH.

The fractions eluted by the first system consisted mainly of triglycerides and the phenolic lipid (I). A solution of this fraction in 10 ml of $CHCl_3$ was deposited on a column (2 × 30 cm) of KSK silica gel (100-150 mesh) that had been treated by a method we have described previously [8]. The column was washed with 100 ml of

$CHCl_3$ and then successively with mixtures of $CHCl_3$ and MeOH in relative amounts of 50:1, 40:1, 30:1, 20:1, and 10:1 (300 ml each). Fractions with a volume of 15 ml were collected and were analyzed by TLC in the $CHCl_3$–MeOH (20:1) and $CHCl_3$–acetone (5:1) systems. The fractions eluted by $CHCl_3$–MeOH (40:1) contained the chromatographically pure lipid (I) with R_f in the above-mentioned solvent systems 0.5 and 0.85, respectively, mp 90-94°C.

UV spectrum [in MeOH; λ_{max}, nm (ε)]: 274 (1280), 280 (1240).

IR spectrum (ν_{max}, cm^{-1}): 3320 (ν_{HO}), 2918 (ν_{CH} antisym), 2855 (ν_{CH} sym), 1629, 1602, and 1512 ($\nu_{C=C}$ arom.), 1470 (δ_{CH} antisym), 1385 (δ_{CH} sym in CH_3), 1200 (ν_{CO}), 828 and 697 (γ_{CH} arom), 718 (ρCH_2),

Acetyl Derivative (II). A solution of 20 mg of the lipid (I) in 0.3 ml of pyridine was treated at 20°C with 0.3 ml of Ac_2O and the mixture was left for 12 h at the same temperature, after which it was evaporated in vacuum. The residue was deposited in 1 ml of CH_2Cl_2 on a column containing 5 g of silica gel; 20 ml of CH_2Cl_2 eluted 20 mg of the acetate (II), R_f 0.65 (CH_2Cl_2), 0.45 (hexane–CH_2Cl_2 (1:1)); mp 62-67°C.

IR spectra (ν_{max}, cm^{-1}); 1785 ($\nu_{C=O}$), 1620 and 1591 ($\nu_{C=C}$), 1222 (ν_{CO}).

For the PMR spectrum and the mass spectrum, see Tables 1 and 2.

Benzoyl Derivative (III). A solution of 5 mg of the lipid (I) in 0.3 ml of pyridine was treated with 30 mg of benzoic anhydride. The mixture was left at 20°C for 24 h and was then treated with 0.2 ml of water and left at 20°C for 2 h, after which it was dissolved in 5 ml of $CHCl_3$. This solution was washed with water, with saturated $NaHCO_3$ solution, and again with water, and was evaporated to dryness. The residue was deposited in 1 ml of hexane–CH_2Cl_2 (3:1) on a column containing 5 g of silica gel; 20 ml of the same solvent system eluted the dry benzoate (III), R_f 0.9 [in CH_2Cl_2, 0.75 (hexane–CH_2Cl_2 (1:1)]; mp 60-68°C.

IR spectrum (ν_{max}, cm^{-1}: 1742 ($\nu_{C=O}$), 1618, 1598, 1480 ($\nu_{C=C}$ arom.).

For the mass spectrum, see Table 2.

Methyl Ether (IV). To a solution of 20 mg of the lipid (I) in 2 ml of MeOH was added an excess of a solution of CH_2N_2 in ether. The mixture was left at 20°C for 5 h and evaporated to dryness, and the residue in 1 ml of CH_2Cl_2 was deposited on a column containing 5 g of silica gel; 20 ml of CH_2Cl_2 eluted 20 mg of the dimethyl ether (IV), R_f 0.85 [hexane–CH_2Cl_2 (1:1)]; mp 56-61°C.

UV spectrum [heptane; λ_{max} nm (ε)]: 273 (1160), 279 (1185).

IR spectra (ν_{max}, cm^{-1}): 2918 (ν_{CH}, antisym), 2852 (ν_{CH} sym), 1614 and 1600 ($\nu_{C=C}$ arom), 1208 (ν_{CO}), 822 and 697 (γ_{CH} atom.), 718 (ρ_{CH_2}).

For the PMR and mass spectrum, see Tables 1 and 2.

SUMMARY

From the total cell lipids of the nitrogen-fixing bacterium Azotobacter chroococcum 92 a previously unknown lipid has been isolated which has been identified as a 1-alkyl-3,5-dihydroxybenzene. The alkyl chains of the main components of the lipid fraction have a normal structure and contain 19 and 21 carbon atoms.

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