PENTAKETIDE METABOLITES OF MUTANTS AND THE BIOSYNTHESIS OF MELANIN IN Verticillium tricorpus

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UDC 547.656+576.809.8+632.428

From mutants of the fungus Verticillium tricorpus Isaac., having various genetic blocks in the biosynthesis of melanin, we have isolated and identified scytalone, flaviolin, 3,3'-biflaviolin, 2-hydroxyjuglone, vermelone, and 1,8-dihydroxynaphthalene, which, in combination with the results of complementation analysis, has enabled the main stages of the biosynthesis of melanin in V.tricorpus to be established. The results on the inhibition of the biosynthesis of melanin by tricyclazole in natural isolates of V. tricorpus indicates that the initital stages of the biosynthesis of melanin in V. tricorpus up to the formation of 1,3,8-trihydroxynaphthalene are identical with those of Verticillium dahliae.

We have previously [1] reported the results of a study of melaninogenesis by Verticillium dahliae Kleb., which permitted the new stages of a proposed scheme for the biosynthesis of melanin in this fungus [2] to be confirmed and supplemented.

In the present paper we consider the physicochemical properties and biosynthesis of the pigment of dormant structures of natural isolates of another phytopathogenic fungus of the genus Verticillium – Verticillium tricorpus Issac., and also information on the chemical structure of substances produced by its mutants.

Six soil (index P) pigmented isolates of V. tricorpus and 27 mutants of V. tricorpus with disturbed pigment formation were used. In natural isolates of V. tricorpus, the microsclerotia and the permanent dauermycellium are black and the chlamydospores are dark brown. The dormant structures of the mutants have the following colors: white (9 mutants) – als (albino structures); dark red (7 mutants) – chs (cherry structures); brown (5 mutants) – brs (brown structures); and olive (6 mutants) – ols (olive structures). In this paper the collection names of the mutants are given.

The pigment isolated from dormant structures of natural isolates of V. tricorpus, just like the melanin from the microsclerotia of V. dahliae used for comparison, were precipitated, when an alkaline extracted was acidified, in the form of a dark brown flocculant precipitate insoluble in water and in organic solvents, and it was declorized by oxidizing agents ($NaOCl$ and H_2O_2), gave a positive reaction with $FeCl_3$ for polyphenols, and did not pass through Cellophane when its alkaline solution was dialyzed; the last-mentioned fact shows its high molecular weight.

In the UV spectra of an alkaline solution of the pigment from V. tricorpus, just like those of the melanin from V. dahliae, there were no absorption maxima in the 400-600 nm region. The IR spectrum of the pigment from V. tricorpus was similar to that of the melanin from V. dahliae [3]. The ESR spectra of the V. tricorpus pigment and of the V. dahliae did not differ with respect to the magnitude of the g-factor and the width of the signal. The dependence of the amplitude of the signal on the SHF power fed showed an extremum the position of which was identical in all the samples investigated. It is obvious that the unpaired electrons of the V. tricorpus pigment and of the V. dahliae melanin are present in similar environments, and therefore it may be assumed that the pigment of the dormant structures of V.tricorpus, just like that of V. dahliae, is melanin and, consequently, their biosynthesis

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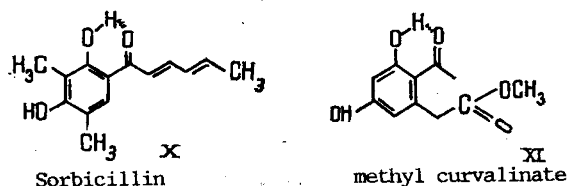
0009-3130/85/2104-0455$09.50 © 1986 Plenum Publishing Corporation

may take place similarly, i.e., through the intermediate formation of pentaketides, as has been established for V. dahliae [1, 2].

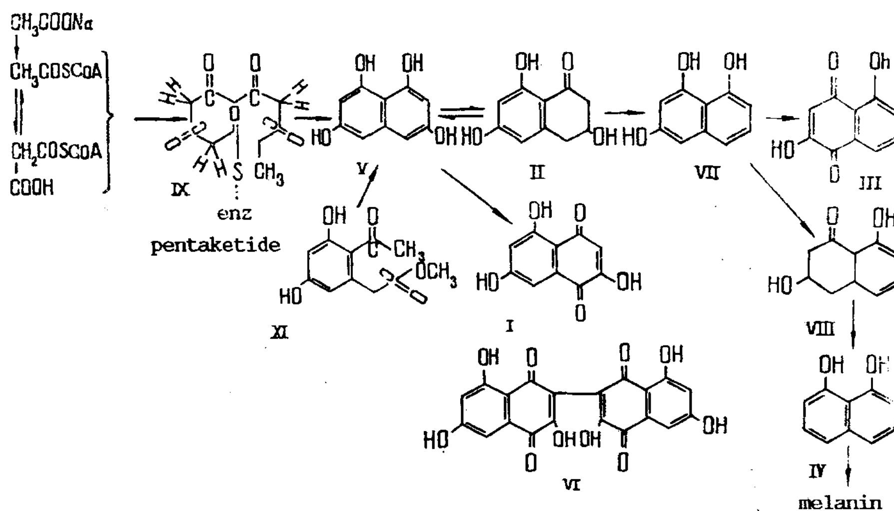
To test this hypothesis we used the V. tricorpus mutants mentioned above with blocks in various stages of the biosynthesis of melanin (melanin-deficient mutants). The pentaketides of the mutants were analyzed by thin-layer chromatography (TLC) on Silufol plates in solvent system 1-3. The chromatographic mobilities (R_f) of the substances present in the extracts were compared with the R_f values of model samples of pentaketides: 2,5,7-trihydroxy-1,4-naphthoquinone (flaviolin) (I), 3,6,8-trihydroxy-3,4-dihydronaphthalen-1(2H)-one (scytalone) (II), 2,5-dihydroxy-1,4-naphthoquinone (2-hydroxyjuglone) (III), 1,8-dihydroxynaphthalene (IV), and 3,3'-bi-2,5,7-trihydroxy-1,4-naphthoquinone (3,3'-biflaviolin) (VI). The metabolites were isolated by the methods of column chromatography (GC) and preparative thin-layer chromatography (PTLC) on Chemapol silica gels L 100/160 and L 5/40 in solvent systems 1-3. The chemical structures of the compounds isolated were established on the basis of their physicochemical properties and chromatographic and spectral characteristics (UV, IR, ^1H and ^{13}C NMR, and mass spectra) and a comparison of them with literature figures [4, 5].

In cultures of the als-mutants forming colonies with colors ranging from golden yellow to dark orange (als 1 – als 9) there were no pentaketide intermediates of the biosynthesis of melanin.

We have established that the color of the colonies of the als-mutants is apparently connected with the presence of an orange pigment in the substrate mycellium. A comparison of melting points and of the chromatographic (R_f) and spectral (UV, IR, PMR, and mass spectra) characteristics of the pigment isolated and those of a model sample – the hexaketide metabolite sorbicillin (X) obtained from a culture of the fungus Penicillium chrysogenium [6] showed their identity.



Also of interest is the presence in the extracts from cultures of the als-mutants of V. tricorpus of a substance identical in its qualitative reactions (with $FeCl_3$ and H_2SO_4) and its chromatographic (R_f), spectral (UV and IR spectra) characteristic with a model compound – methyl curvulinate (IX) obtained from Curvularia geniculata [7] which is, according to [8], an inductor of spore formation in lower fungi of the genera Aspergillus, Pencillium, Helminthosporium, Alternaria, Ascophyta, Sclerotinia, Trichoderma, Curvularia, etc. A simple method exists for obtaining 1,3,6,8-tetrahydroxynaphthalene (V) from (XI) [9]. As is well knwon [1, 10], substance (V) is an intermediate in the biosynthesis of melanin in V. dahliae and some other imperfect fungi. Consequently, the presence in V. tricorpus of



Pathway of the biosynthesis of melanin in Verticillium tricorpus

a previously undescribed block in the biosynthesis of melanin at the stage of the conversion of (XI) into (V) (see scheme) may be assumed.

The addition of exogenous scytalone (II) to cultures of some als-mutants of V. tricorpus — for example, als 1 and als 3 — caused a blackening of the colonies and the formation of a pigment the physicochemical properties of which did not differ from those of melanin extracted from natural isolates. The other als-mutants did not possess this property. Consequently, mutants of V. tricorpus with this phenotype in relation to the reaction to the addition of (II) and the absence of pentaketides in acetone extracts are similar to the alm-mutants of V. dahliae [1].

The capacity of the als-mutants of V. tricorpus for producing melanin when (II) is added to a culture of the fungus and the absence of pentaketides metabolites in acetone extracts from these mutants indicates a blockage in them, just as in the alm-mutants of V. dahliae, of the biosynthesis of melanin at the stage preceding the formation of (V). This compound is a common intermediate in the biosynthesis both of (II) and of (I) [5]. In view of information [11] on the biosynthesis of (II), it may be assumed that the blockage of the biosynthesis of melanin in the als-mutants of V. tricorpus takes place either at the stage of the conversion of (XI) into (V), as mentioned above, or at the stage of the conversion of the hypothetical "pentaketide" (IX) into (V).

The main pentaketides of seven mutants with the phenotype chs 1 — chs 7 are (II) and (I). In particular, the V. tricorpus mutants chs 1, chs 2, chs 4, and chs 7 synthesized mainly (I), but in the mutants chs 2 and chs 4 considerable amounts of a substance with R_f 0.25 in system 1 were detected, in addition. In the PMR spectrum of this substance in deuteroacetone there were two doublets, at 6.68 and 7.18 ppm, with a splitting within the doublets of 2.5 Hz. The UV, IR, and mass spectra of the substance corresponded to those described previously for 3,3'-bi-2,5,7-trihydroxy-1,4-naphthoquinone (3,3'-biflaviolin) (VI) [6]. In their capacity for accumulating (I) and (VI), therefore, these mutants are similar to the chm mutants (group II) of V. dahliae described previously [1] in which the blocking of the biosynthesis of melanin takes place at the stage of the conversion of (V) into (II). Consequently, in V. tricorpus this stage also exists in the biosynthesis of melanin. The presence in the biosynthesis of melanin by V. tricorpus of a stage of the conversion of (V) into (II) is also confirmed by the the fact that the systemic fungicide tricyclazole (5-methyl-[1,2,4]-triazole[3,4-b]benzothiazole) at a concentration of 10 μg/ml in the cultivation medium of V. tricorpus causes the synthesis of a large amount of (I) [12] and also, as we have established, of (VI), while other pentaketides are practically absent. Thus, tricyclazole blocks the biosynthesis of melanin in V. tricorpus at the stage of the reduction of (V) to (II). In this case, (I) can be formed only form (V), as has been established for V. dahliae [1, 10]. The increase in the amount of (VI) in the culture media of the imperfect fungi V. dahliae, Thielaviopsis basicola, etc., when the biosynthesis of melanin in them is blocked by tricyclazole in certain concentrations has been shown previously [13]. But we are the first to have established the presence of V. tricorpus mutants synthesizing (VI) and the capacity of natural V. tricorpus isolates subjected to the action of tricyclazole for secreting considerable amounts of (VI) into culture media.

The mutants chs 3, chs 5, and chs 6 synthesized (II) and a very small amount of (I); consequently they are similar to the mutants brm 1, brm 3, and chm (group 1) of the fungus V. dahliae described in the literature [1, 4] in which the stages of the conversion of (II) into 1,3,8-trihydroxynaphthalene (VII) are blocked.

Compound (III) has been isolated from an acetone extract of the cultures of five mutants having the phenotypes brs 1 — brs 5 and has been identified. With respect to the presence of this metabolite in culture media, the mutants of this group are similar to the mutants brm 2 [2] and brm of V. dahliae described in the literature [1]. The naphthoquinone (III) is a product of the autooxidation of (VII) [4], and therefore, by analogy with V. dahliae, it is possible to assume the presence in the above-mentioned V. tricorpus mutants of a genetic block at the stage of the conversion of (VII) into (VIII).

The treatment of natural isolates of V. tricorpus P-24 and P-26 with tricyclazone in concentrations of 0.1-1.0 μg/ml led to the inhibition of the synthesis of melanin in them. Compound (III) was extracted from cultures of these isolates and identified. Its accumulation as the main pentaketide metabolite shows a blockage by tricyclazone of the conversion of (VII) into (VIII) [12].

When (VIII) was added to a culture of als-mutants of V. tricorpus they synthesized (IV), just like the alm-mutants of V. dahliae [14]. This indicates the presence of stage of the conversion of (VIII) and (IV) in the biosynthesis of melanin by V. tricorpus just as in the case of V. dahliae [10].

In acetone extracts of the six mutants having the phenotypes ols 1 – ols 6, the presence of a substance with a chromatographic mobility similar to that of the model compound (IV) was established in solvent systems 4-6. When an acetone extract of the als-mutants was evaporated in vacuum, an aqueous residue was obtained, the acidification of which to pH 2-3 led to the formation of a pigment identical in its physicochemical properties with the substance obtained as the result of the autooxidation of (IV) [15]. Consequently, in the isolation of the substance presumed to be (IV) the stage of acidifying the aqueous extract followed by extraction with ethyl acetate that is present in the isolation of the pentaketides [1, 4] was avoided. In place of this, the aqueous residue was lyophilized and was then worked up as described in the Experimental part. The UV and mass spectra of the compound isolated were similar to those described previously for (IV) [15], and the ^{13}C NMR spectrum of its solution in $(CD_3)_2CO$ contained six signals in the low-field region. In the proton spectrum, two groups of signals of the ABC type at 6.60 and 7.2 ppm corresponded to it, and so did a singlet at 10.1 ppm which disappeared when the spectrum was recorded in deuteromethanol because of intermolecular exchange between the –OH and –OD groups, which corresponds completely to the structure (IV).

We have established that the addition of (II) and (IV) to als-mutants of V. tricorpus leads to the biosynthesis of melanin in them. This is in harmony with the analogous capacity of these metabolites for forming melanin in alm-mutants of V. dahliae [6].

Summarizing the results presented above and bearing literature information [1, 2, 9, 11] in mind, the biosynthesis f melanin in V. tricorpus can be represented in the form of the scheme, from which it can be seen that the main stages of the biosynthesis of melanin in V. tricorpus are identical with those in V. dahliae.

EXPERIMENTAL

The conditions for recording the UV, IR, NMR, and mass spectra were similar to those described in [17].

Analytical thin-layer chromatography was performed on Silufol plates in the following solvent system: 1) ether–benzene–formic acid (50:50:1); 2) chloroform–acetone (9:1); and 3) acetone–methanol (9:1); and paper chromatography in systems 4) 15% acetic acid; 5) 0.5 N HCl; and 6) an aqueous solution of ammonia saturated with butanol. The plates were visualized in iodine vapor and ammonia, and the substances on the paper chromatograms were detected visually or after treatment with an ethanolic solution of iodine.

The natural isolates of V. tricorpus (P-19, P-21, P-24, P-26, P-35, and P-43) were obtained from the collection of fungi of the genus Verticillium in the Department of General Genetics of the Cotton Plant of the Academy of Sciences of the Tadzhik SSR, and isolates of Penicillium chrysogenium and Curvularia geniculata from the collection of lower fungi in the D. K. Zabolotnii Institute of Microbiology and Virology of the Academy of Sciences of Ukrainian SSR.

Mutants were induced from the strains P-24 and P-26 by UV radiation as described previously [18], and were divided into phenotypically homogeneous groups according to the results of complementation analysis.

Cultures of natural isolates and mutants of the fungus V. tricorpus were grown in Petri dishes or solid agarized Czpek medium in the dark at 24-26°C for 7-10 days. Tricyclazole (EL-291) was added to the nutrient medium of the natural isolates before the inoculation of the fungus in the form of an ethanolic solution, and the corresponding amount of ethanol was added to the control.

Isolaton of Melanin from the Dormant Structures of the Fungus. The agar with the fungus that had grown on it in Petri dishes was cut into small pieces, placed in a flask, and covered with hot water (5 volume soft water per volume of agar), and the mixture was carefully stirred and filtered. The fungus on the filter was washed repeatedly with hot water to eliminate agar and was then treated with a small amount of acetone to eliminate water from its surface and was dried in air and homogenized in 150 ml of 1 M KOH, and the resulting

mixture was heated under reflux in an atmosphere of nitrogen [3]. After filtration through a triple paper filter, the dark brown filtrate was acidified with HCl (1:1, v/v) to pH 2-3. The dark brown precipitate was separated off by centrifugation, washed with distilled water, and lyophilized, and in this way melanin was obtained. Part of the precipitate before lyophilization was redissolved in 1 M KOH at room temeprature in an atmosphere of nitrogen, giving an alakaline solution of melanin.

Acetone extracts of cultures of the als-, chs- and brs-mutants and of natural isolates treated with various concentrations of tricyclazole were obtained as described earlier for *V. dahliae* [1].

Preparation of Acetone Extracts of the ols-Mutants. The agar in the Petri dishes was cut into small pieces which were placed in a flask, covered with acetone (5 volumes of solvent per volume of agar) and were left in the shaking machine for 8 h; the mixtures were then filtered and the acetone was distilled off in vacuum in a IR-1M evaporator. The aqueous residues so formed were lyophillized.

The isolation of the metabolites from acetone extracts of the cultures of the mutants and cultures of wild isolates of *V. tricorpus* treated with tricyclazole and their identification was carried out as described previously [1, 19]. The UV, IR, PMR, and mass spectra of the compounds (I), (II), and (III) isolated were identical with those described in the literature [4, 5].

Sorbicillin (X) from *V. tricorpus* formed orange prisms with mp 121-123°C from a mixture of ether and pentane (1:1) at −20°C. A model sample of (X) from *P. chrysogenium* had mp 122-125°C. The R_f value of substance (X) in TLC on Merck silical gel + 13% of gypsum in the $CHCl_3$–C_2H_5OH (94.6) system was 0.62. When X was revealed on chromatographic plates with a solution of $FeCl_3$ in C_2H_5OH/H_2O it appeared in the form of a black spot.

UV spectrum, λ_{max} (MeOH), nm: 320, 380.

IR spectrum (cm^{-1}): 3580, 3440-3100, 2970, 2910, 2850, 1640, 1615, 1560.

PMR spectrum (in $CDCl_3$), ppm: t, 2.13; t, 2.20; d, 5.30; dd, 7.43; s, 13.60.

Mass spectrum, m/z (%): 234 (6), 233 (13), 232 (82, M^+), 231 (20), 218 (15), 217 (100), 191 (38), 189 (17), 175 (18), 165 (76), 164 (34), 138 (18), 136 (59), 135 (11), 109 (10), 91 (16), 83 (21), 79 (17), 77 (19), 67 (21), 65 (19), 55 (17), 53 (16), 43 (26), 41 (43).

Isolation and Identification of 3,3'-Bi-2,5,7-trihydroxy-1,4-naphthoqunione (3,3'-Biflaviolin) (VI). An acetone extract of the mutant chs 2 was chromagographed on 15 × 15 cm Silufol plates in system 1, and the zone with a substance having R_f 0.25 was scraped from the plates, placed in a microcolumn, and eluted with acetone. After the removal of the solvent, the substance present in the residues was recrystallized from ethyl acetate. Substance (VI) did not melt below 350°C.

UV spectrum, λ_{max} (ethanol/NaOH), nm: 290, 370, 420, 555.

PMR spectrum (in deuteroacetone), ppm: d 6.68, J = 2.5 Hz, 2 H; d, 7.18, J = 2.5 Hz, 2 H; s, 12.51, 2 H, exchange with D_2O.

Mass spectrum (380°C), m/z (%): 207 (3), 206 (15), 167 (15), 150 (13), 149 (100).

The isolation of 3,8-dihydroxy-3,4-dihydronaphthalen-1 (2H)-one (vermelone) (VIII) was carried out from chs-mutants producing scytalone to a culture of which compound (VII) obtained by the method of Stipanovic and Bell [5] had been added. The (VIII) was isolated from the acetone extract by preparative TLC on glass plates (18 × 24 cm) coated with Chemapol silica gel 5/40 + 13% of gypsum in solvent system 1 (R_f 0.31). When the plates were treated with 1% $FeCl_3$, compound (VIII) was scraped from the plate and transferred to a chromatographic column, and the (VIII) was eluted with ether. After recrystallization from cyclohexane, crystals of (VIII) with mp 91-93° were obtained.

UV spectrum (in ethanol) λ_{max}, nm: 259, 333.

The IR spectrum was identical with that described for (VIII) in [19].

PMR spectrum (in deuteroacetone), ppm: 3.00-3.30; m, 4.32; t, 7.45, J = 7.3 Hz; d, 8.75, J = 7.3 Hz; s 12.42.

Mass spectrum, m/z (%): 178 (98), 161 (20), 160 (100), 135 (25), 134 (98), 132 (44),

131 (20), 107 (13), 106 (62), 105 (32), 104 (28), 103 (16), 78 (54), 77 (44), 63 (13), 52 (19), 51 (31).

The conversion of 3,8-dihydroxy-3,4-dihydronaphthalen-1 (2H)- one into 1,8-dihydroxynaphthalene (IV) by the chs-mutants of *V. tricorpus* was carried out by the method described in [5].

Isolation and Identification of 1,8-Dihydroxynaphthalene (IV). To isolate (IV), acetone extracts from cultures of ols-mutants of *V. tricorpus* were used. The lyophilized aqueous fraction of the acetone extract was treated with acetone again, and this was evaporated in a rotary evaporator. The concentrated extract was separated by ascending paper chromatography in system 6. In system 6, compound (IV) has R_f 0.65.

UV spectrum (in ethanol), λ_{max}, nm: 305, 320, 333.

PMR spectrum (in CD_3OD), ppm: 6.6, 7.2.

^{13}C NMR spectrum (in deuteroacetone), ppm: 107.4 (C-2 and C-7); 113.6 (C-9); 118.0 (C-4 and C-5); 125.3 (C-3 and C-6); 135.7 (C-10); and 152.5 (C-1 and C-8).

Mass spectrum (100°C), m/z (%): 160 (M^+, 100), 131 (24), 132 (15), 115 (10), 114 (61), 113 (10).

The capacity for stopping the biosynthesis of melanin in als-mutants of *V. tricorpus* on the addition of (II) and (IV) to cultures of them was studied by the method described previously [1].

SUMMARY

The physicochemical properties of pigments of the dormant structures of *Verticillium tricorpus* have been studied and their identity with those of the melanin of *V. dahliae* has been shown. Scytalone, flaviolin, 2-hydroxyjuglone, 3,3'-biflaviolin, vermelone, and 1,8-dihydroxynaphthalene have been isolated from mutants of the fungus *V. tricorpus* having various genetic blocks in the biosynthesis of melanin and identified. The substances accumulating in a culture of the fungus *V. tricorpus* under the action of the systematic fungicide tricyclazole on it have been identified. The main stages of the biosynthesis of melanin in *V. tricorpus* have been established for the first time by the methods of biochemical genetics and their identity with those for *V. dahliae* has been shown.

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LIPOPOLYSACCHARIDES OF MARINE BLUE-GREEN ALGAE

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UDC 547.917+632.4

The lipopolysaccharides (LPSs) have been isolated from two blue-green algae, Oscillatoria hilderbrandtii and Nostoc sp. by the method of phenol—water extraction. The LPSs contain polysaccharide and lipid components. The polysaccharide fraction from O. hildebrandtii consists predominantly of 1,3-bound rhamnose residues with a substituent in the second position. The rhamnose residues in the polysaccharide fraction of Nostoc are connected by 1,3- and 1,2-glycosidic bonds. The lipid components of the LPSs from O. hilderbrandtii and Nostoc consist of palmitic and steric acids, with glucosamine and glucose.

Blue-green algae, which occupy an intermediate position between lower plants and microorganisms, are a basically new source of lipopolysaccharides (LPSs) distinguished by high serological activity and low toxicity [1]. We have previously [1-3] given the chemical characteristics of the LPSs of a number of fresh-water algae. In the present paper we consider the LPSs isolated for the first time from the marine blue-green algae Oscillatoria hildebrandtii and Nostoc sp. We must point out the existence of large amounts of the biomass of both algae under natural conditions.

The LPSs were isolated from both algae by Westphal's method [4]. Further purification with the aid of ultracentrifugation led to purified preparations of the LPSs from O.hildebrandtii (LPS-OS) and from Nostoc sp. (LPS-N) with yields of 1-3%. Analysis showed that both LPSs were characterized by low contents of 2-keto-3-deoxyoctonic acid (KDO), which is a characteristic component of the LPSs of Gram-negative bacteria. This is due to a simple bond between the lipid and carbohydrate moieties of the LPSs of blue-green algae. We have observed a similar situation for other species of blue-green algae [1-3]. Nevertheless, the mild acid hydrolysis of LPSs with dilute acetic acid gave the polysaccharide and lipid components in both cases.

In the complete hydrolysate of both lipids, glucosamine was detected as the main saccharide component with a small amount of glucose, which may be a component of the carbohydrate chain of the LPS and be located at the juction of the lipid and carbohydrate compponents. The fatty acid compositions of both lipids were characterized by the presence of residues of palmitic ($C_{16:0}$) and stearic ($C_{18:0}$) acids. They were identified with the aid of gas-liquid chromatography (GLC) in the form of their methyl esters. It is interesting to note the absence of residues of β-hydroxy acids in the lipids investigated, as we have recorded previously [1-3] for other blue-green algae. This is an important difference of the lipid components of the LPSs of blue-green algae from the lipid A of Gram-negative bacteria, which is probably responsible for the low toxicity of the LPSs of the blue-green algae and opens up the possibility of using them as nonspecific immunostimulators [5].

The complete acid hydrolysis of both LPSs showed that their carbohydrate chains included residues of rhamnose, glucose, galactose, mannose, arabinose, xylose, glucosamine, and glucuronic acid, the amount of rhamnose considerably exceeding the amount of any of the other monosaccharide residues. Furthermore, the LPS-OS contained residues of fucose and of methylated sugars- 2- and 3-O-methylrhamnose, which were absent from the LPS-N.

The polysaccharide components of both LPSs obtained as the result of mild acid hydrolysis with subsequent elimination of the lipid were separated by chromatography on column of Sephadex G-75 into two fractions: a high-molecular-weight fraction (PS-1) and a low-molecular-

Pacific Ocean Institute of Bioorganic Chemistry, Far Eastern Scientific Center, Academy of Sciences of the USSR, Vladivostok. Translated from Khimiya Prirodnykh Soedinenii, No. 4, pp. 493-496, July-August, 1985. Original article submitted September 17, 1984.

0009-3130/85/2104-0461$09.50 © 1986 Plenum Publishing Corporation