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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 components. 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-

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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

weight fraction (PS-2). A similar phenomenon is observed in the case of bacterial LPSs when gel filtration on Sephadexes of their polysaccharide components obtained after mild acid hydrolysis leads to the production of a high-molecular-weight O-specific polysaccharide and an oligosaccharide core [6]. Glucose and rhamnose were identified in hydrolysates of the PS-2 fractions obtained from both polysaccharides. These fractions were not investigated subsequently, since the greatest interest was presented by the high-molecular-weight polysaccharides (PS-1) which may correspond to the O-specific polysaccharides of Gram-negative bacteria. They were arbitrarily designated as PS-OS and PS-N, as corresponding to the LPS-OS and LPS-N. Complete acid hydrolysis showed that the PS-OS and PS-N included residues of the following monosaccharides in the given respective amounts: rhamnose (47 and 51%), glucose (9 and 14%), galactose (4 and 8%), mannose (4 and 10%), arabinose (1 and 12%), xylose (8 and 5%), and fucose (11 and 0%). In addition, residues of 2-O- and 3-O-methyl-L-rhamnose (5 and 11%) were detected in the PS-OS. They were identified by demethylation with the aid of hydrobromic acid [7] and also from the results of the GLC and mass spectrometry of the corresponding polyol acetates.

Thus, PS-OS and PS-N are complex heteropolysaccharides with a predominant content of rhamnose.

In the periodate oxidation of PS-OS and PS-N, 0.33 and 0.5 mole, respectively, of periodate per anhydro unit were consumed. The polysaccharides obtained were reduced with the aid of tetrahydroborate to the corresponding polyalcohols in yields of 70 and 50%, respectively. Rhamnose was identified in a hydrolysate of the polyalcohols obtained. In the case of PS-OS, 3-O-methylrhamnose was also detected. These results indicate that the rhamnose residues are minaly included in the carbohydrate chains of both LPSs with the aid of 1→3 glycosidic bonds or are present in part at points of branching. The other monosaccharides either occupy terminal positions or are included in a linear chain by 1→2, 1→4-, or 1→6-bonds. it is not excluded that because of their small amounts some of them were not detected in the hydrolysate by standard methods.

To determine the nature of the bond between the monosaccharide residues we carried out the exhaustive methylation of the PS-OS and PS-N. The methylated polysaccharides were subjected to methanolysis followed by analysis of the methylated sugars with the aid of GLC and chromato-mass spectrometry. Unfortunately, because of the small amount of the individual methylated sugars in the mixture, it was possible to make a strict identification only of the rhamnose derivatives. In the case of PS-OS, derivatives of 2,3,4-tri-, 2,4,-di, and 4-O-methylrhamnose were identified. This confirmed the presence of a 1→3-bond between the rhamnose residues and, in addition, the fact that some residues of rhamnose or its monomethyl derivatives were present in terminal positions and a number of rhamnose residues formed points of branching of the main carbohydrate chain of the PS-OS. In the case of the PS-N, 2,3,4-tri-, 2,4-, and 3,4-di-O-methylrhamnoses were identified, which showed the presence of terminal and 1→2- and 1→3-bound residues in the carbohydrate chain of the polysaccharide. Consequently, the main carbohydrate chains of both polysaccharides contained rhamnose residues linked predominantly by 1→3-glycosidic bonds.

From analyses of the LPSs of blue-green algae of the genus Phormidium [1], Microcystis aeruginosa [2], Spirulina platensis [3], Oscillatoria hilderbrandtii, and Nostoc sp. it can be stated that all the LPSs studied have similar monosaccharide compositions. Some of them (Sp. platensis, M. aeruginosa, and Ph. laminosus) are distinguished by a high glucose content. The LPS from M. aeruginosa is also characterized by an elevated mannose content, and the polysaccharide components of the LPSs from Nostoc sp.and O. hildebrandtii by a high rhamnose content.

In the monosacchardie compositions, all the LPSs isolated showed a similarity to the O-antigens of Gram-negative bacteria but differed from the latter by a low content of a lipid component, by the absence of β-hydroxy acids from them and also by the low content of KDO in the carbohydrate moiety.

EXPERIMENTAL

The general experimental conditions have been given by us previously [1-3].

Blue-Green Algae. The biomass of the alga O. hildebrandtii was collected in the Timor Sea, Indian Ocean in a region with the coordinates 10°36' S and 129°17' E during the expedition of the Scientific-Research Ship "Professor Bogorov" in 1981. Nostoc sp. (it was impossible to determine the species) was collected in the littoral band of a number of islets of

the South Shetland Islands in January, 1980, by V. A. Nicolaev (Botanical Institute of the Academy of Sciences of the USSR, Leningrad), who was a member of an Antarctic expedition.

The cells were fixed with ethanol and the biomass was extracted with ethanol and acetone and dried in the air. The air-dry raw material was used in the investigation.

Isolation of the LPSs. The biomass of the blue-green algae (100 g) was extracted and worked up by Westphal's method [4]. Crude LPS fractions were obtained, the yields of LPS-OS and LPS-N being 3 and 2%, respectively. A 1% solution of the crude LPS-OS fraction (3 g) was freed from impurities by centrifugation at 105,000 × g for 4 h. Purified LPS-OS was obtained with a yield of 2.6 g; it contained 48% of carbohydrates and 6% of protein. A 0.5% solution of the crude LPS-N fraction (2 g) was freed from impurities by centrifugation at 160,000 × g for 1 h. Purified LPS-N was obtained with a yield of 1 g; it contained 60% of carbohydrates and 5% of protein.

Complete Acid Hydrolysis. An LPS or a PS (5 mg) was heated with 1 N sulfuric acid (3 ml) in a sealed tube at 100°C for 5-6 h. The mixture was neutralized with barium carbonate, deionized with KU-2 ion-exchange resin in the $[H^+]$ form, and evaporated. The monosaccharides were identified by PC and GLC.

Partial Hydrolysis. A. The LPS-OS (260 mg) was heated with 10% acetic acid (20 ml) at 100°C for 3 h. The precipitate of lipid was separated off by centrifugation and it was washed with water and acetone. Yield 50 mg. The dry residue was extracted with a mixture of chloroform and methanol (2:1 by volume). The extract was evaporated to dryness. The yield of purified lipid was 12 mg (4.6% on the weight of the LPS).

The supernatant after the separation of the lipid was additionally purified by extraction with chloroform. The aqueous phase was lyophilized. A mixture of polysaccharide fractions was obtained with a yield of about 200 mg.

The mixture of polysaccharides (100 mg) was chromatographed on a column (1.8 × 92 cm) of Sephadex G-75 with elution by pyridine-acetate buffer (pH 4.2). The separation was monitored by the phenol/sulfuric acid method. The fractions corresponding to the peaks on the elution curve were combined and lyophilized. This gave a high-molecular-weight fraction (PS-OS) with a yield of 25 mg (25% of the charge to the column): $[\alpha]_D$ −36° (c 0.6; water), carbohydrate content 70%, protein content 1%, nucleic acid content 0.3%. In addition, a low-molecular-weight fraction (PS-2) was obtained with a yield of 30 mg and a monosaccharide fraction with a yield of 35 mg.

B. The LPS-N (60 mg) was heated with a 1% solution of acetic acid (10 ml) for 5 h. The precipitate of lipid was separated by centrifugation and was purified as described above. The yield of lipid was 1.8 mg (3% on the weight of the LPS).

The supernatant (40 mg) was chromatographed on the same solumn. The fraction issuing after the free volume of the column was lyophilized. This gave PS-N with a yield of 14 mg and PS-2 with a yield of 8 mg.

Smith Degradation. PS-OS or PS-N (10 mg) was oxidized with a 0.015 M solution of sodium metaperiodate (10 ml) at room temperature. The consumption of periodate was 0.33 mole per anhydro unit for the PS-OS and 0.5 mole for the PS-N. The polyaldehyde obtained was treated with sodium tetrahydroborate (10 mg) at 20°C for 16 h. The excess of tetrahydroborate was decomposed by the addition of acetic acid to pH 5. The polyalcohol was evaporated to dryness with methanol. The residue was dialyzed and lyophilized. The yield of the OS polyalcohol was 7 mg and that of the N polyalcohol 5 mg. Each polyalcohol was hydrolyzed with 1 N sulfuric acid at 100°C for 5 h and the products were investigated by PC and GLC.

Analysis by the Methylation Method. The PS-OS and the PS-N (10 mg in each case) were methylated by Hakomori's method. The completeness of methylation was determined from the absence of a hydroxyl absorption band in the IR spectrum. The methylated compounds were heated in sealed tubes with 1 N hydrogen chloride in absolute methanol at 100°C for 10 h, and the resulting methylglycosides were acetylated with acetic anhydride in pyridine and analyzed by GLC and chromato-mass spectrometry in comparison with authentic samples.

Hydrolysis of the Lipid. The lipid (5 mg) was hydrolyzed with 4 N hydrochloric acid (3 ml) at 100°C for 4 h. The mixture was diluted with water and extracted with chloroform. The aqueous phase was investigated by PC. The chloroform extract was dried over

anhydrous sodium sulfate and evaporated. The residue was treated with 0.5 N hydrogen chloride in absolute methanol at 100°C for 7 h. The fatty acid methyl esters obtained were analyzed by GLC.

SUMMARY

1. Lipopolysaccharides the carbohydrate components of which consists of heteropolysaccharides have been isolated from the marine blue-green algae Oscillatoria hildebrandtii and Nostoc sp.

2. Rhamnose residues are included in the carbohydrate chain of the LPS from O. hildebrandtii by 1→3-bonds with substituents in the second position.

3. The rhamnose residues in the carbohydrate chain of the LPS from Nostoc sp. are linked by both 1→2-and by 1→3-glycosidic bonds.

4. The lipid components from O. hildebrandtii and Nostoc sp. are constructed of residues of palmatic and stearic acids and glucosamine and glucose.

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FLAVONOIDS OF THE EPIGEAL PART OF Rhodiola rosea.

II. STRUCTURES OF NEW GLYCOSIDES OF HERBACETIN AND OF GOSSYPETIN

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UDC 547.972

The structure of four new flavonol glycosides isolated from the epigeal part of Rhodiola rosea have been established: 7-O-α-L-rhamnopyranosylgossypetin (rhodiolgin), 8-O-β-D-glucopyranosyl-7-O-α-L-rhamnopyranosylgossypetin (rhodiolgidin), 8-O-β-D-glucopyranosol-7-O-α-L-rhamnopyranosylherbacetin (rhodionidin), and 3-O-β-D-glucopyranosyl-8-O-β-D-xylopyranosylherbacetin (rhodalidin). The properties of the previously undescribed incomplete methyl ethers of herbacetin and gossypetin obtained during the structural analysis of the glycosides have been studied. It has been found that diazomethane methylates the 5-OH groups in the diglycosides investigated.

We have previously [1] reported the isolation from the epigeal part of Rhodiola rosea L. (Sedum rosea, roseroot stonecrop), family Crassulaceae of rhodionin (V), rhodalin (VI), and four new flavonoid glycosides – rhodiolgin (I), rhodiolgidin (II), rhodionidin (III), and rhodalidin (IV). In the present paper we have given information on the determination of the structures of the new compounds (I-IV).

On the basis of their PMR spectra and the results of acid hydrolysis, compound (I) was assigned to the monoglycosides (rhamnoside) and compounds (II-IV) to the diglycosides, containing, respectively, rhamnose and glucose (II and III) and xylose and glucose (IV). The

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All-Union Scientific-Research Institute of Medicinal Plants, Moscow. Translated from Khimiya Prirodnykh Soedinenii. No. 4, pp. 496-507, July-August, 1985. Original article submitted September 3, 1984.

 0009-3130/85/2104-0464$09.50