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OLIGOMERIC PROANTHOCYANIDIN GLYCOSIDES OF *Rhodiola pamiroalaica*. II

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In a continuation of investigations of proanthocyanidins of the roots of Rhodiola pamiroalaica, *we have isolated proanthocyanidins RP-3 and RP-4. Their compositions, structures, and relative configurations have been investigated: RP-3 is 7-O-(6-O-galloyl-β-*D*-Glcp)-3-O-galloyl-(−)-epigallocatechin-(4β—8)-[(−)-epicatechin-(4β—8)-(3-O-galloyl-(−)-epigallocatechin)]$_2$-(4β—8)-[5-O-(β-*D*-Glcp $\xrightarrow{6}$ *O-β-*D*-Glcp)-(+)-catechin], and RP-4 is 7-O-(6-O-galloyl-β-*D*-Glcp-3-O-galloyl-(−)-epigallocatechin-(4β—8)-[3-O-galloyl-(−)-epicatechin]-(4β—8)-[3-O-galloyl-(−)-epigallocatechin]-(4β—8)-[3-O-galloyl-(−)-epicatechin-(4β—8)-[3-O-galloyl-5-(β-*D*-Glcp $\xrightarrow{6}$ *O-β-*D*-Glcp)-(−)-epigallocatechin*

As reported previously [1], two proanthocyanidins, RP-1 and RP-2, have been isolated from *Rhodiola pamiroalaica A. Bor.* In the present paper we give the results of a study of the physicochemical properties of compounds RP-3 and RP-4 isolated from the same plant.

UV and IR spectra showed that substance RP-3 was a proanthocyanidin. In its ^{13}C NMR spectrum compound R-3 showed signals characteristic for epigallocatechin, epicatechin, and catechin, and for glucose and gallic acid residues (Table 1).

Resonance signals of the C-5, C-7, and C-9 atoms of the phloroglucinol nucleus appeared in the form of a broadened signal at 153.2—155.4 ppm [2]. An intense signal at 144.7 ppm related to C-3′ and C-5′ of ring *B*. The C-4′ atom of gallocatechin is screened, and, as a result of a diamagnetic shift, resonated at 132.4 ppm. At the same time the presence of signals at 116.2 and 120.3 ppm, relating to the C-2, C-5, and C-6 atoms, respectively, showed that RP-3 included a catechin block [2, 3].

In the spectrum there is the characteristic signal for gallic acid at 139.1 ppm, relating to C-4′[3, 4]

The signals of the C-2 atoms in fragments of compound RP-3 appeared at 81.2, 77.1, and 75.8 ppm, which unambiguously showed the existence of both 2,3-*cis*- and 2,3-*trans*- stereochemistries of the flavan-3-ones in this compound [5—9]. Signals relating to C-2 and C-3 of the heterocycle were located at 75.8 and 74.3 ppm, respectively, and, consequently, some of the "upper" blocks were galloylated. The "bottom" block in proanthocyanidin RP-3 was not galloylated, as was shown by the chemical shifts (CSs) of the signals of carbon atoms C-2, C-3, and C-4 (81.2, 65.9, and 27.1 ppm, respectively) [10]. The chemical shift of the C-10 atoms of the blocks, observed at 101.3 ppm, showed that the interflavan bonds in this proanthocyanidin were of the C-4→C-8 type [1].

It is interesting to note that the signals of glucose residues and of glucose residues with galloylated hydroxy groups appeared in the spectrum.

The carbohydrate residue was galloylated in the sixth position, as was witnessed by the presence of a signal at 62.3 ppm relating to a substituted C-6 atom of glucose. The presence of signals at 60.4 and 64.1 ppm relating to the C-6 atoms indicated that two of the glucose residues were linked by a 1—6-bond.

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TABLE 1. Chemical shifts (ppm) of the Signals of the Carbon Atoms in the ^{13}C NMR Spectrum of Proanthocyanidin RP-3

Carbon atom	Fragment of RP-3				Glucose	Glucose	Glucose	Gallate
	a	b	c	d				
2	75.8	77.1	75.8	81.2				
3	74.3	71.3	74.3	65.9				
4	34.6	36.4	34.6	27.1				
6	95.4	95.4	95.4	95.4				
8	93.1	106.8	106.8	106.8				
10	101.3	101.3	101.3	101.3				
5,7,9			153.2-155.4					
1'	130.5	130.5	130.5	130.5	101.3	101.3	101.3	120.3
2'	109.9	116.2	109.9	116.2	75.8	75.8	75.8	109.9
3'	144.7	144.7	144.7	144.7	77.1	77.1	77.1	144.7
4'	132.4	144.7	132.4	144.7	71.7	71.7	72.9	139.1
5'	144.7	116.2	144.7	116.2	75.8	75.8	77.1	144.7
6'	109.9	120.3	109.9	120.3	64.4	62.3	60.4	109.9
COO								164.6
								166.4

The chemical shifts of C-1, C-3, and C-5 of the glucose residues showed that the anomeric centers had the β-configuration [11].

A number of chemical transformations were carried out to establish the chemical structure of RP-3. Its alkaline cleavage in an atmosphere of nitrogen formed phloroglucinol (**1**), protocatechuic acid (**2**), and gallic acid (**3**). The phloroglucinol was formed from rings *A* and the phenolic acids from rings *B*, while the fragments consisting of the C-3 and C-4 atoms of rings *C* were isolated in the form of acetic acid [12].

The interflavan C—C bonds were cleaved by the acid hydrolysis of RP-3. This led to (+)-catechin (**4**), cyanidin (**5**), delphinidin (**6**), glucose (**7**), and galloylglucose (**8**). On the thiolytic cleavage of RP-3 in the presence of thiophenol and acetic acid three compounds were obtained. (+)-Catechin (**4**) was formed from the bottom part of the molecule and was identified from its melting point and its IR and PMR spectra. The upper blocks gave a mixture of thioethers (**9** and **10**), which were cleaved catalytically with Raney nickel. The substances obtained as a result of this reaction were identified from their physicochemical and spectral characteristics as (-)-epigallocatechin 3-O-gallate (**11**) and (-)-epicatechin (**12**).

The presence of a catechin residue in the bottom position was also confirmed by the chemical shifts of the C-2, C-3, and C-4 atoms in the ^{13}C NMR spectrum of RP-3.

Enzymatic cleavage in the presence of the enzyme β-glucosidase and subsequent acid hydrolysis of a permethylate of RP-3 showed that the carbohydrate residues consisted of molecules of glucose and galloylglucose linked with the aglycon by a β-glycosidic bond. The presence of β-glucose residue was also confirmed by the chemical shifts of the C-1, C-3, and C-5 atoms in the ^{13}C NMR spectrum (see Table 1).

On the basis of the spectral and chemical results, we propose for RP-3 the structure and relative configuration of 7-O-(6-O-galloyl-β-*D*-Glc*p*)-3-O-galloyl-(-)-epigallocatechin-(4β—8)-[(-)-epicatechin-(4β—8)-(3-O-galloyl-(-)-epigallocatechin)]$_2$-(4β—8)-[5-O-(β-*D*-Glc*p* $\xrightarrow{6}$ O-β-*D*-Glc*p*)-(+)-catechin].

According to its UV and IR spectra, RP-4 was also a proanthocyanidin. In order to investigate features of its spatial and chemical structure we studied the ^{13}C NMR spectrum of this compound.

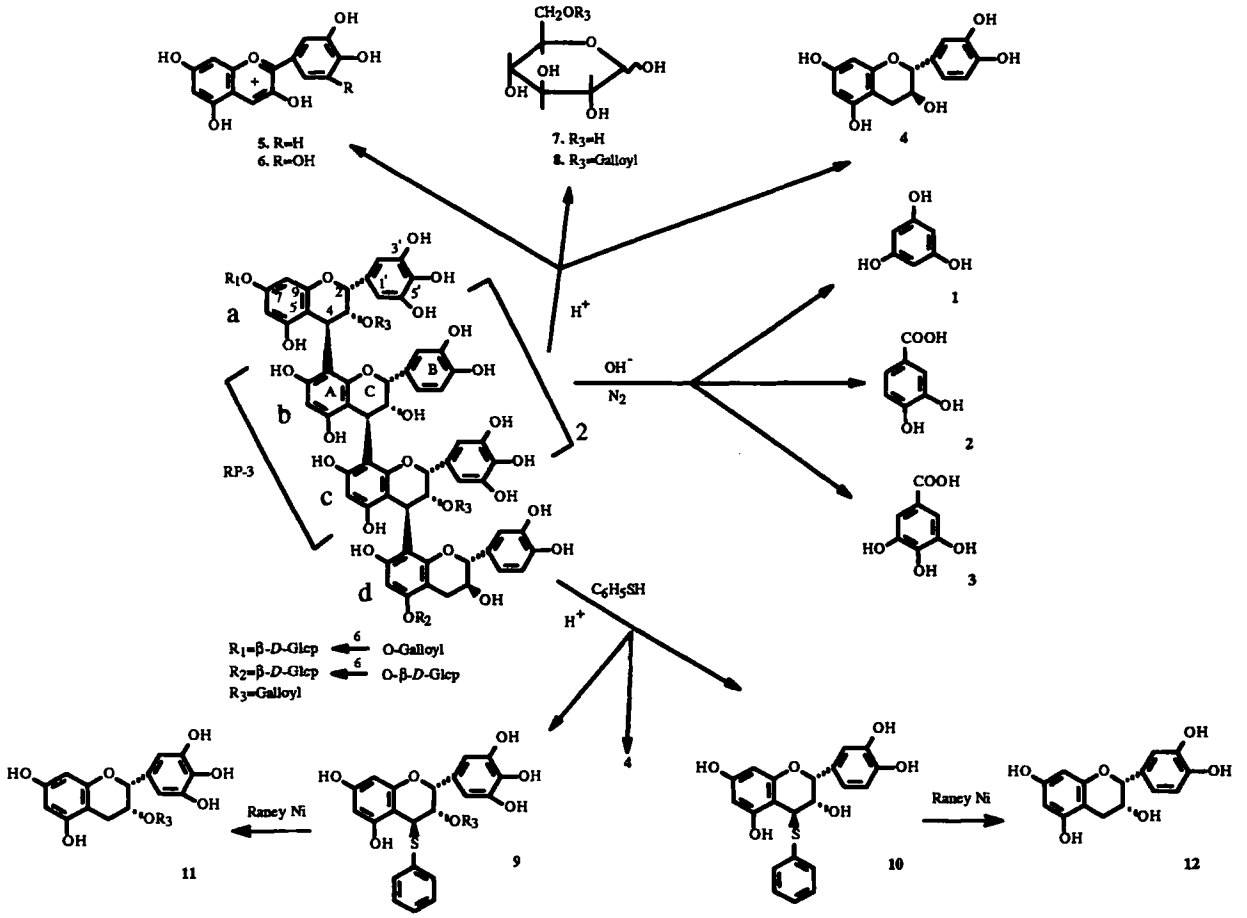
The ^{13}C NMR spectrum of RP-4 showed the signals of galloylated catechins, of glucose, and of galloylglucose (Table 2). Resonance signals in the 151.2—153.7 ppm region related to C-5, C-7, and C-9 of the phloroglucinol residues of rings *A*. A consideration of the CSs of the carbon atoms of rings *B* of this compound enabled us to detect in it the presence of epicatechin and epigallocatechin systems.

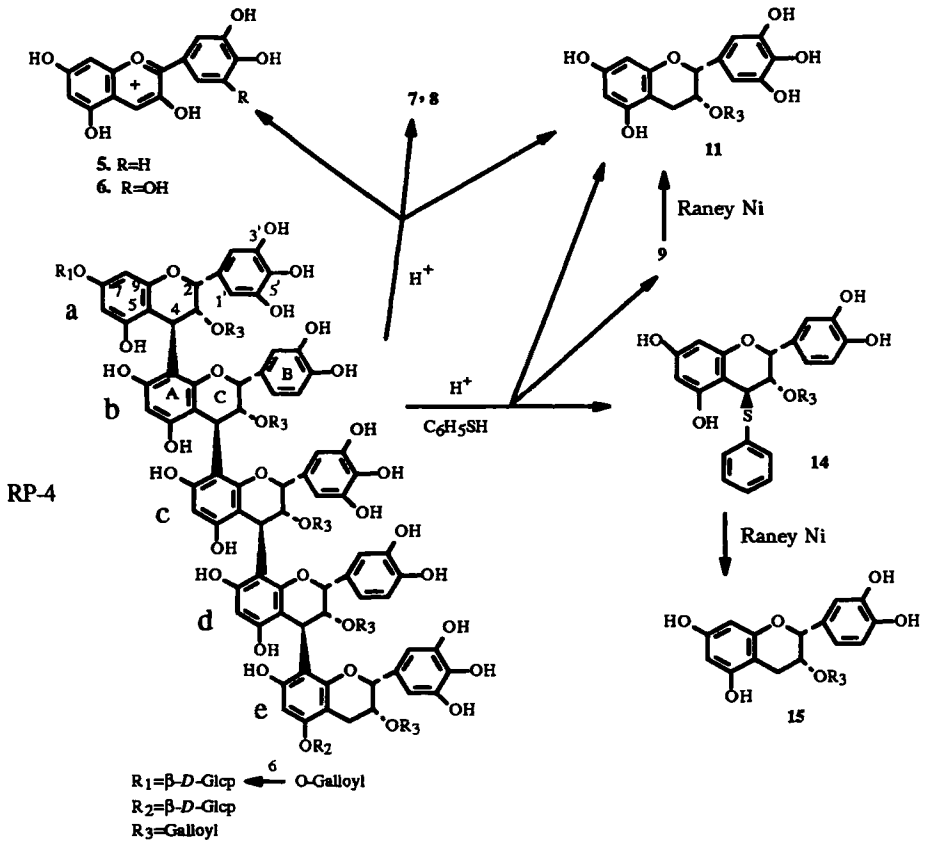
Intense signals at 142.6 ppm related to C-3′ and C-4′ of ring *B* of the epicatechins (**12**) and also to C-3′ and C-5′ of rings *B* of the epigallocatechins. The C-4′ atoms of the epigallocatechins resonated at 131.8 ppm. Resonance signals at 117.4 and 119.2 ppm related to the unsubstituted carbon atoms of rings *B* of the catechins. Intense signals at 111.6 ppm were characteristic for the C-2′ and C-6′ atoms of rings *B* of the epigallocatechins and of gallic acid.

TABLE 2. Chemical Shifts (ppm) of the Signals of the Carbon Atoms in the ^{13}C NMR Spectrum of Proanthocyanidin

Carbon atom	Fragment of RP-4					Glucose	Glucose	Gallate
	a	b	c	d	e			
2	74.0	74.0	74.0	74.0	76.2			
3	74.0	74.0	74.0	74.0	69.2			
4	33.4	33.4	33.4	33.4	26.4			
6	97.0	94.0	94.0	94.0	94.0			
8	94.2	106.8	106.8	106.8	106.8			
10	100.1*	103.4*	103.4*	103.4*	103.4*			
5,7,9			151.2-153.7					
1'	130.2	130.2	130.2	130.2	130.2	103.4*	103.4*	119.2
2'	111.6	117.4	111.6	117.4	111.6	74.0	74	111.5
3'	142.6	142.6	142.6	142.6	142.6	76.2	76.2	142.6
4'	131.8	142.6	131.8	142.6	131.8	71.9	72.5	138.8
5'	142.6	117.4	142.6	117.4	142.6	76.2	74.0	142.6
6'	111.6	119.2	111.6	119.2	111.6	60.4	62.7	111.6
COO								162.5
								164.9

*The signals may be interchanged.





The absence of a weak-field signal of C-2 carbon atoms (81—85 ppm) and its appearance at 74.0—76.2 ppm permitted the assumption that the C-2 and C-3 asymmetric centers had the 2,3-*cis*-configuration.

Resonance in the regions of 74.0, 33.4, 76.2, 69.2, and 26.0 ppm of signals relating to the C-2, C-3, and C-4 atoms of the heterocycles showed that all the blocks were galloylated in the C-3 position. Signals with chemical shifts in the region of 100.1—103.4 ppm, relating to the C-10 atoms, were characteristic for proanthocyanidins with C-4–C-8 interflavan bonds.

In the ^{13}C NMR spectrum of proanthocyanidin RP-4, there were, in addition, the signals of the carbon atoms of sugar residues. A study of this group of signals showed that one glucose residue at C-6 was bound to gallic acid, the characteristic signal of which appeared at 62.7 ppm, while another glucose residue, not acylated, was responsible for the signal of a C-6 atom at 60.4 ppm.

To determine the degrees of oxidation of rings *B* we carried out an alkaline fusion, as a result of which compounds (**1**—**3**) were formed, while acid hydrolysis gave compounds (**5**—**8**, and **11**).

Thiolytic cleavage followed by reduction of the resulting thioethers showed that all the blocks of RP-4 were galloylated. The carbohydrate moiety in proanthocyanidin RP-4 was analyzed by the methylation method. The permethylate formed was hydrolyzed in an acid medium. This gave 2,3,4-tri-O-methyl-*D*-glucopyranose and 2,3,4,6-tetra-O-methyl-*D*-glucopyranose.

On the basis of its spectral and chemical characteristics, we propose for RP-4 the structure and relative configuration of 7-O-(6-O-galloyl-β-*D*-Glcp)-3-O-galloyl-(−)-epigallocatechin-(4β—8)-[3-O-galloyl-(−)-epicatechin]-(4β—8)-[3-O-galloyl-(−)-epigallocatechin]-(4β—8)-[3-O-galloyl-(−)-epicatechin-(4β—8)-[3-O-galloyl-5-O-(β-*D*-Glc*p* $\xrightarrow{6}$ O-β-*D*-Glc*p*)-(−)-epigallocatechin.

EXPERIMENTAL

The UV spectra of the proanthocyanidins and their derivatives were taken in alcoholic solution on a Perkin-Elmer Lambda-16 instrument, and IR spectra on a Perkin-Elmer System 200 FT-IR instrument in tablets with potassium bromide.

^{13}C NMR spectra were obtained on a Tesla BS 567 A instrument (25 MHz for ^{13}C nuclei) in Me_2CO-d_6—D_2O (1:1)

solution under conditions of complete suppression of spin-spin interaction with protons; internal standard TMS, δ-scale, duration of the ninety-degree pulse 8 ms, delay 1, number of accumulations: for RP-3, 14900, and for RP-4, 16500. Concentration of the substance 20%. Molecular masses were determined on a MOM 3170 ultracentrifuge (speed 50000 rpm, temperature 20°C, angle 30°, speed 8000 rpm) and by gel filtration on a calibrated column of Sephadex LH-20. To check the homogeneity of the substances we used PC and TLC on Silufol plates. The elementary analyses of all the compounds corresponded to the calculated values.

Isolation of the Total Proanthocyanidins. Comminuted roots of *Rh. pamiroalaica* (6 kg) were extracted six times with 36% aqueous alcohol. The extracts obtained were combined and evaporated in vacuum at 50°C. The concentrated extract was diluted with water (1:3) and was treated exhaustively with ethyl acetate and *n*-butanol. This yielded 216 and 330 g of dry residues from the corresponding fractions. After evaporation, the aqueous residue yielded a total of 1464 g of proanthocyanidins.

Separation of the Proanthocyanidins. The butanolic extract (70 g) was mixed with cellulose (70 g), transferred to a column of microcrystalline cellulose (6 × 140 cm, 1680 g), and eluted with chloroform—methanol, methanol, and methanol—water, with the collection of 100-ml fractions. Homogenous fractions were combined and rechromatographed on a column of Sephadex LH-20 (5 × 160 cm). The homogeneity of the fractions was monitored by TLC.

Proanthocyanidin RP-3 (0.950 g was isolated), composition $C_{136}H_{120}O_{70}$, M 2872. UV spectrum (nm): λ_{max} 210, 245, 275, λ_{min} 256. IR-spectrum: (ν_{max}, cm^{-1}): 3321, 1695, 1611, 1539, 1448, 1339, 1207, 1145, 1103, 1035, 826, 765, 734. For details of the ^{13}C NMR spectrum, see Table 1.

Proanthocyanidin RP-4 (0.870 g was isolated), composition $C_{129}H_{106}O_{67}$, M 2726. UV spectrum (nm): λ_{min} 208, 245, 274, 305, λ_{min} 257. IR-spectrum: (ν_{max}, cm^{-1}): 3240, 1695, 1615, 1540, 1450, 1338, 1210, 1150, 1100, 1033, 825, 735. For details of the ^{13}C NMR spectrum, see Table 2.

Alkaline Cleavage of RP-3. With the passage of a slow current of nitrogen, a 20-ml four-necked round-bottomed flask was charged with 80 mg of RP-3, and then 5 ml of a 50% solution of caustic soda was added. With constant stirring, the lower part of the flask was immersed in a bath with a low-melting metallic alloy at a temperature of 150—160°C. The bath temperature was raised over 5 min to 230°C and then the reaction mixture was rapidly cooled by immersing the flask in ice water and acidified with 20% sulfuric acid. The contents of the flask were diluted with water and extracted with ethyl acetate. The ethyl acetate extract was dried with anhydrous sodium sulfate, and the solvent was distilled off. The residue was chromatographed on a column of polyamide. Three compounds were obtained, which were identified by their physicochemical and spectral characteristics as phloroglucinol (**1**), protocatechuic acid (**2**), and gallic acid (**3**).

The Alkaline Cleavage of RP-4 was conducted by the method described for RP-3. Three compounds were obtained: phloroglucinol (**1**), protocatechuic acid (**2**), and gallic acid (**3**).

Acid Cleavage of RP-3. A solution of 150 mg of the substance in 6 ml of ethanol was treated with 2.5 ml of a 2 N solution of hydrochloric acid, and was heated in the water bath under reflux in a current of nitrogen for 2 h. The reaction mixture was diluted with water and extracted with ethyl acetate. The extract was washed with sodium bicarbonate solution and dried with anhydrous sodium sulfate, and the solvent was distilled off. The residue was chromatographed on a column of Sephadex LH-20. This gave 7 mg of (+)-catechin, $C_{15}H_{14}O_6$, mp 178—180°C, $[\alpha]_D^{22}$ +21° (*c* 0.51; acetone—water, 1:1), cyanidin (**5**), delphinidin (**6**), glucose (**7**), and glucose 6-O-gallate (**8**), $C_{13}H_{16}O_{10}$ mp 137—138°C $[\alpha]_D^{22}$ +22° (*c* 0.32; acetone).

Acid Cleavage of RP-4. The method described above was used to treat 100 mg of the substance. The residue was chromatographed on a column of Sephadex LH-20 with elution by 80% ethanol. This led to (−)-epigallocatechin 3-O-gallate (**11**), cyanidin (**5**), delphinidin (**6**), glucose (**7**), and a monogalloylglucose, M^+ 332, mp 135—137°C, $[\alpha]_D^{22}$ +26° (*c* 0.18; acetone).

Thiolytic Cleavage of RP-3. A mixture of 350 mg of RP-3 and 5 ml of thiophenol was treated with 3 ml of acetic acid and 10 ml of ethanol and was left at room temperature for 48 h. During the first 10 h, the course of the reaction was monitored by TLC every hour. Then the reaction mixture was concentrated and the oily residue obtained was chromatographed on Sephadex LH-20 and eluted with ethanol.

This gave 11 mg of (+)-catechin and 210 mg of an amorphous substance consisting of a mixture of two thioethers.

Cleavage of the Thioethers. The thioethers (210 mg) were mixed with 4 ml of ethanol—acetic acid (9:1), the catalyst — Raney nickel — was added, and the reaction mixture was kept at 50°C for 1 h. Then it was filtered, and the filtrate was concentrated and chromatographed on Sephadex LH-20 with elution by 80% ethanol. Two compounds were obtained: (−)-

epigallocatechin 3-O-gallate (**11**) and epicatechin (**12**).

Thiolytic Cleavage of RP-4. The method described above was used to cleave 110 mg of this substance. We obtained (-)-epigallocatechin 3-O-gallate (**11**) and thioethers. Reductive degradation of the thioethers led to (-)-epigallocatechin 3-O-gallate (**11**) and (-)-epicatechin 3-O-gallate (**15**).

Enzymatic Hydrolysis of RP-3 and RP-4. The enzyme β-glucosidase was added to a solution of 0.1 g of one of the glycosides in 10 ml of water. The reaction mixture was placed in a thermostat and kept at 30°c for 6 h. Polyphenols were precipitated with a solution of lead acetate, and in each case glucose was detected in the filtrate by paper chromatography.

Methylation of RP-3. A solution of 0.150 g of the glycoside in 20 ml of dimethyl sulfoxide was treated with 0.2 g of sodium hydride, and the mixture was stirred at room temperature for 1 h. Then 5 ml of methyl iodide was added in drops, and stirring was continued for another 4 h, after which the reaction mixture was poured into ice water (50 ml) and extracted with a mixture of ethyl acetate and chloroform. The extract was treated with sodium thiosulfate, washed with water, and dried with anhydrous sodium sulfate. After the solvent had been distilled off, the residue was methylated similarly another five times. The reaction products were separated by column chromatography, and 0.03 g of amorphous permethylate was obtained.

Hydrolysis of the Permethylate of RP-3. The permethylate (0.03 g) was dissolved in 8 ml of aqueous methanol (1:1) containing 5% of sulfuric acid, and the solution was heated on the water bath for 8 h. Then the reaction mixture was neutralized with barium carbonate, the precipitate was filtered off, and the filtrate was evaporated to dryness. The residue was purified on a chromatographic column. Thus gave 0.005 g of methylated carbohydrates. The methylated sugars were identified by TLC in comparison with similar specimens and proved to be 2,3,4-tri-O-methyl-*D*-glucopyranose and 2,3,4,6-tetra-O-methyl-*D*-glucopyranose.

Methylation of RP-4. Methylation, subsequent hydrolysis of the permethylate, and the detection of the methylated sugars were carried out by the method described above. The products were, again, 2,3,4-tri-O-methyl-*D*-glucopyranose and 2,3,4,6-tetra-O-methyl-*D*-glucopyranose.

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