BACTERIAL ANTIGENIC POLYSACCHARIDES

COMMUNICATION 3. DATA ON THE STRUCTURE OF THE REPEATING UNIT OF THE POLYSACCHARIDE CHAIN OF LIPOPOLYSACCHARIDE FROM TYPE 6 Shigella dysenteriae

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The monosaccharide composition of all nine serological types was established in the course of studying the bacterial O-antigenic lipopolysaccharides (LPS) of the Sh. dysenteriae subgroup [1]. Here the LPS of serological types 3 and 6, which contain heptose, ketodesoxyoctonic acid, glucose, galactose, and galactosamine, were assigned to one chemotype. When the structure of the polysaccharide chain of the LPS from type 3 Sh. dysenteriae was studied it was found that, together with the enumerated sugars, a new acid monosaccharide enters into the composition of the LPS [2]. Data are given in the present paper on the structure of the repeating unit of the polysaccharide chain of the LPS from type 6 Sh. dysenteriae, from which it conclusively follows that the LPS of serological types 3 and 6 belong to different chemotypes.

The LPS was isolated from the dry bacterial cells of type 6 Sh. dysenteriae by extraction with hot aqueous phenol and subsequent precipitation of the nucleic acids with cetavlon [hexadecyltrimethylammonium bromide] [3]. After purification by ultracentrifuging the yield of the LPS was 4% of the weight of the dry cells. The obtained LPS is active toward autoclaved baterial culture in the passive hemagglutination test with O-antiserum. The LPS was subjected to hydrolysis with dilute AcOH solution and was split into the lipid and carbohydrate components. After removal of the lipid by centrifuging the carbohydrate portion was separated into two fractions by gel-chromatography on Sephadex G-50. The first, high-molecular-weight fraction represented a polysaccharide chain with an oligosaccharide "cortex" attached to it; its yield was 25% of the LPS weight. The second, oligosaccharide fraction apparently represented the LPS "cortex" and the low-molecular-weight fragments of the polysaccharide chain.

The obtained polysaccharide moved as one zone toward the anode during paper electrophoresis. Its IR spectrum had the absorption bands of the carboxyl (1730 cm^{-1}, weak) and acetamido (1650 and 1560 cm^{-1}) groups. The signals of the carbohydrate protons and the protons of the acetamido group (δ 1.96 ppm) were present in the NMR spectrum of the polysaccharide; the absence of other signals indicated that the acid fragment of the polysaccharide was neither a pyruvate nor an aliphatic acid moiety.

To determine the monosaccharide composition the polysaccharide was subjected to acid hydrolysis (2 N HCl, 100°, 4 h). A study of the hydrolyzate by paper electrophoresis disclosed the presence of a hexosamine and neutral sugars. The absence among the products of both drastic (2 N HCl) and mild (0.01 N HCl) hydrolysis of compounds that move toward the anode indicated that the acid component of the polysaccharide is also not a uronic acid nor the acid monosaccharide found in the LPS of type 3 Sh. dysenteriae [2]. The acid component was apparently also not the 3-desoxy-pentulosonic acid that was discovered recently in the polysaccharide from the capsule of type 38 Klebsiella [4], since it was not cleaved during the degradation of the LPS with dilute AcOH solution. As a result, it is not excluded that the discovered labile acid component is a new component of the LPS of Gram-negative bacteria.

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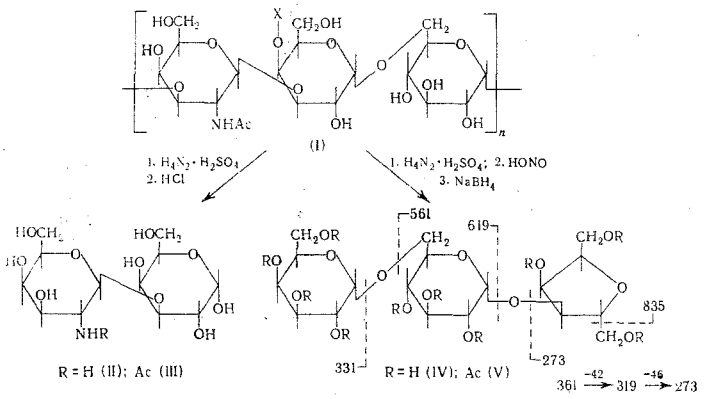
The neutral monosaccharides were identified by paper chromatography (PC) and GLC to be glucose and galactose. The hexosamine was identified on an amino acid analyzer as being galactosamine, and its amount was 22.5%. The amount of D-glucose, determined in the hydrolyzate (2 N HCl) employing glucosooxidase, was 20%. Analysis of this hydrolyzate by GLC after deamination by treatment with HNO_2 [5] led to the identification of 2,5-anhydro-talose, galactose, and glucose in a 1.1:1:1 ratio. The D-configuration of the galactosamine and galactose was established employing D-galactosooxidase (EC 1.1.3.9): incubation of the hydrolyzate with the enzyme leads to the complete oxidation of both monosaccharides. As a result, the polysaccharide from type 6 Sh. dysenteriae contains D-glucose, D-galactose, and N-acetyl-D-galactosamine in a 1:1:1 ratio, and also a new unidentified acid component X.

The data on the structure of the polysaccharide from type 6 Sh. dysenteriae were obtained mainly by the method developed by us [6-9], which involves the selective cleavage of the polysaccharide chain of the hexosaminoglucan via the N-deacetylation of the N-acetyl-hexosamine residues and subsequent acid hydrolysis or deamination. The N-deacetylation of the $NaBH_4$-reduced polysaccharide was effected with hydrazine in the presence of hydrazine sulfate at 105°. The degree of N-deacetylation was determined from the results of the acid hydrolysis of the polysaccharide with free amino groups (see below). The N-deacetylation went to the extent of 70% in 10 h, whereas on the model biosides [6-8] and the polysaccharide from type 3 Sh. dysenteriae [9] it was quantitative under these conditions. Longer treatment (20 h) led to 93% N-deacetylation, although it was accompanied by partial destruction of the polysaccharide; thus only 75% of the taken polysaccharide was eluted when the hydrazinolyzate was subjected to gel chromatography on Sephadex G-50 using a column with a blank volume. The monosaccharide composition of the N-deacetylated polysaccharide is identical with the composition of the starting polysaccharide when based on the results of studying the deaminated hydrolyzate by GLC. A study of the hydrolyzate of the N-deacetylated polysaccharide without deamination led to the detection of only glucose; from this it followed that the galactosamine in the polysaccharide was attached to the galactose, which during hydrolysis is retained as galactosaminyl-galactose (II). Employing an amino acid analyzer, the hydrolyzate of the N-deacetylated polysaccharide was found to contain galactosamine, disaccharide (II), and an unidentified product, which was presumably modified during the hydrazinolysis of the acid component X. The yield of disaccharide (II) as a function of the hydrolysis time is given in the Experimental section. These data served as a base for determining the degree of N-deacetylation of the polysaccharide from the ratio of the yield of disaccharide (II) and galactosamine under the optimum hydrolysis conditions. The N-deacetylated polysaccharide was hydrolyzed (2 N HCl, 100°, 3 h), and the hydrolyzate was subjected to chromatography on a column packed with cationite Dowex 50-W × 8. The neutral monosaccharides were eluted with 0.025 M, while disaccharide (II) was eluted with 0.1 M pyridine–acetate buffer. The unknown component, which was detected using the amino acid analyzer, was not eluted with 0.1 M buffer. Disaccharide (II) when deaminated with HNO_2 is cleaved into galactose and 2,5-anhydro-talose, which were identified by GLC, and consequently is galactosaminyl-galactose. Then disaccharide (II) was subjected to N-acetylation, and the obtained disaccharide (III) was isolated in the pure state by preparative PC.

The hydrolysis of the N-acetylated disaccharide (III) and subsequent deamination led to equal amounts of galactose and 2,5-anhydro-talose. Disaccharide (III) gave a positive Morgan–Elson test [10], and in its chromatographic mobility on paper was identical with 3-O-(2-acetamido-2-desoxy-β-D-galactopyranosyl)-D-galactose, which we had isolated previously from the polysaccharide of type 3 Sh. dysenteriae [9]. The incubation of disaccharide (III) with the β-hexosaminidase from the epididymis of the boar (EC 3.2.1.30) [11] led to its complete cleavage into N-acetyl-galactosamine and galactose. As a result, disaccharide (III) is 3-O-(2-acetamido-2-desoxy-β-D-galactopyranosyl)-D-galactose. From the presented data it follows that the N-acetyl-galactosamine residue in the polysaccharide from type 6 Sh. dysenteriae is attached to the galactose residue in the 3 position.

Then the N-deacetylated and starting polysaccharides were oxidized with $NaIO_4$. A study of the hydrolyzates of both oxidized polysaccharides after deamination by GLC revealed that in both cases the glucose residue is oxidized, while the galactose and galactosamine residues are resistant to oxidation. Consequently, the galactosamine residue bears a substituent in the 3 position. The oxidized polysaccharide was subjected to mild acid hydrolysis under conditions that did not cleave the starting polysaccharide (0.5 N HCl, 20°, 16 h). Here, based on the data of gel chromatography on Sephadex G-50, the oxidized polysaccharide was cleaved completely into oligosaccharide fragments, which could occur in the case where the residue of one of the monosaccharides that compose the linear chain of the polymer is oxidized by the $NaIO_4$. From these data it follows that the glucose residue lies in the linear chain of the polysaccharide; the conclusion was confirmed by the methylation data and by the isolation of oligosaccharide (IV).



Scheme 1

The N-deacetylated polysaccharide was deaminated using a 5% $NaNO_2$ solution in dilute AcOH [6]. This treatment led to rupture of the galactosaminide linkages and a complete cleavage of the polysaccharide into oligosaccharide fragments (data of gel chromatography on Sephadex G-50). Based on the PC data, the deamination product after reduction with $NaBH_4$ represented a mixture of two oligosaccharides with $R_{lactose}$ 0.76 and 1.10. Both oligosaccharides were isolated by preparative PC in the pure state. On acid hydrolysis the more mobile oligosaccharide gave equal amounts of glucose and galactose. The amount of this oligosaccharide was small, and it was not investigated further. Based on the PC and GLC data, the hydrolysis of the main, less mobile oligosaccharide (IV) led to equal amounts of glucose, galactose, and 2,5-anhydrotalitol, i.e., to the same monosaccharides that are formed in the hydrolysis and deamination of the starting polysaccharide. As a result, oligosaccharide (IV) represents a chemically modified repeating unit of the polysaccharide chain of the LPS from type 6 Sh. dysenteriae.

The structure of oligosaccharide (IV) was established on the basis of the mass spectral (MS) data for its full acetate (V) and by methylation. The fragmentation of (V) under electron impact proceeded in the same manner as described for 3-O-(β-D-galactopyranosyl)-2,5-anhydro-mannitol acetate [6], as is shown in Scheme 1. In order to determine the types of glycoside linkages, trisaccharide (IV) and the starting polysaccharide were studied by the methylation method using the Hakomori conditions [12]. The methylated products were subjected to formolysis and hydrolysis; the obtained partially methylated monosaccharides were studied as the polyol acetates by the GLC – MS method (Fig. 1). The identification of the monosaccharides by comparison with authentic samples on the basis of the GLC and MS [13] data is given in Table 1.

The main components of the mixture of partially methylated monosaccharides from the polysaccharide are 2,3,4-tri-O-methyl-glucopyranose and 2,6-di-O-methyl-galactose. The minor peaks correspond to the partially methylated monosaccharides of the "cortex," to which the polysaccharide chain is attached. As a result, the polysaccharide is branched. The absence of completely methylated monosaccharides in the hydrolyzate of the methylated polysaccharide indicates that the unreduced ends of the side chains, attached to the disubstituted galactose, are occupied by the unidentified acid component X.

TABLE 1. Data for Analysis of Polysaccharide and Oligosaccharide (IV) by Methylation

Peaks	Interpretation of substitution of hexitols	Retention times*	Mole ratios	
			polysaccharide	oligosaccharide
A	1, 4, 6-Tri-O-methyl-2, 5-anhydro-talitol	0.5	–	1.0
B	2, 3, 4, 6-Tetra-O-methyl-dulcitol	1.0	0.06	0.9
C	2, 4, 6-Tri-O-methyl-sorbitol	1.56	0.13	–
D	2, 3, 4-Tri-O-methyl-sorbitol	2.0	1.0	1.0
E	3, 4, 6-Tri-O-methyl-dulcitol	2.0	0.1	–
F	2, 6-Di-O-methyl-dulcitol	3.14	1.12	–
G	4, 6-Di-O-methyl-sorbitol	3.24	0.1	–

*The retention times are relative to 2, 3, 5, 6-tetra-O-methyl-dulcitol acetate at 155°.

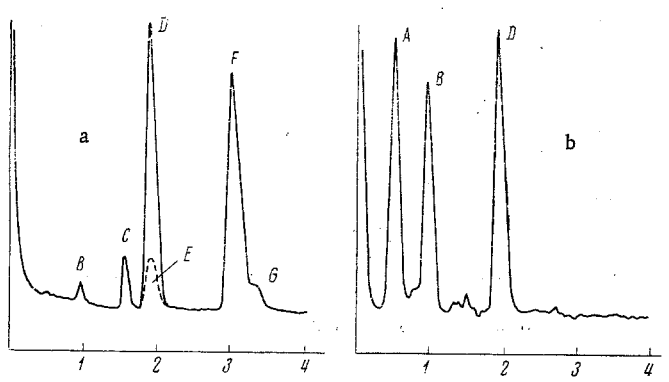


Fig. 1. Gas – liquid chromatogram of partially methylated polyol acetates, obtained from methylated polysaccharide (a) and oligosaccharide (b). The retention times are relative to 2,3,4,6-tetra-O-methyl-dulcitol acetate.

The mixture of methylated monosaccharides, obtained from trisaccharide (IV), was found to contain 1,4,6-tri-O-methyl-2,5-anhydro-talitol, 2,3,4,6-tetra-O-methyl-galactopyranose, and 2,3,4-tri-O-methyl-glucopyranose in an equimolar ratio. The appearance of 2,3,4,6-tetra-O-methyl-galactopyranose in place of 2,6-di-O-methyl-galactose, which was detected in the hydrolyzate of the methylated polysaccharide, is explained by the elimination of the galactosamine residue as 2,5-anhydro-talose during deamination, which leads to freeing one of the two substituted OH groups. The second substituted OH group is apparently freed when the acid component is cleaved during the N-deacetylation and deamination of the polysaccharide. Based on these data, trisaccharide (IV) is galactopyranosyl-(1 – 6)-glucopyranosyl-(1 – 3)-2,5-anhydro-talitol. A number of conclusions regarding the structure of the starting polysaccharide follows from this. First, trisaccharide (IV) is practically the sole product of the selective cleavage of the polysaccharide, which is direct proof for the presence of repeating oligosaccharide units. Second, the galactosamine and glucopyranose residues are found in the linear chain of the polysaccharide, while the acid component is attached as a branch directly to the galactose residue in the 4 position. Third, the presence of galactopyranose on the unreduced end of trisaccharide (IV) testifies that the galactose is present in the polysaccharide as the pyranose form, which did not follow directly from the methylation of the polysaccharide itself. The last problem that had to be solved was to determine the configuration of the glycoside linkages. The β-configuration of the galactosaminide linkage followed from the data for the enzymatic hydrolysis of disaccharide (III). To determine the configurations of the glucoside and galactoside linkages the acetylated trisaccharide (V) was oxidized with CrO_3 in AcOH as described in [14, 15]. A study of the hydrolyzate of the oxidized trisaccharide (V) disclosed that the glucopyranose and galactopyranose residues are resistant to oxidation, and consequently are attached by α-glycoside linkages. The practically complete oxidation of the 2,5-anhydro-talitol residue proved unexpected, but this fact was confirmed experimentally using an authentic sample of the full acetate of 2,5-anhydro-talitol.

The optical rotation values of the polysaccharide, $[\alpha]_D$ + 56.5°, and of trisaccharide (IV), $[\alpha]_D$ + 74°, are found to be in agreement with the determined configurations of the glycoside linkages.

The combination of the presented data makes it possible to assign structure (I) to the chemical repeating unit of the polysaccharide chain of the O-specific LPS of typs 6 Sh. dysenteriae.

EXPERIMENTAL

The PC was carried out on FN-11 paper in the systems: 6 : 4 : 3 n-butanol – pyridine – water (system A) and ethyl acetate – CH_3COOH – HCOOH – water (system B). The paper electrophoresis was carried out in 0.025 M pyridine – acetate buffer at a potential gradient of 28 V / cm. The sugars were detected using alkaline $AgNO_3$, acid aniline phthalate, or KIO_4 and benzidine. The gel-chromatography was carried out in pyridine – acetate buffer with pH 4.5 (10 ml of CH_3COOH and 4 ml of pyridine in 1 liter of water); the output curves were constructed from the data of the reaction with phenol – sulfuric acid [16]. The GLC method described in [5] was used to analyze the aminosugars and determine the ratios of the monosaccharides. The GLC was carried out on a Pye Unicam 104 instrument using a 90 × 0.4 cm column packed with 3% ECNSS-M deposited on Gaschrome Q (100-120 mesh), while the GLC – MS was carried out on a

Varian Gnom MAT-111 instrument using the same phase and support. The NMR spectra were taken on an XL-100 spectrometer in D_2O solution at 90°, while the mass spectra were taken on a Varian CH-6 instrument. The optical rotation was determined on a Perkin – Elmer 141 polarimeter. The solvents were evaporated in vacuo at 40°.

Isolation of O-Specific LPS and Polysaccharide. The bacterial cells of type 6 Sh. dysenteriae, Tyakht strain No. 458-679, were grown on nutrient medium containing casein hydrolyzate by the depth method with aeration. The cells, dried in succession by acetone and ether, were extracted with hot aqueous phenol, the aqueous layer was dialyzed, and the nucleic acids were precipitated with cetavlon as described in [3]. The obtained LPS was purified by ultracentrifuging (105,000 g, 4 h). The LPS (500 mg) was hydrolyzed with 60 ml of dilute AcOH solution (pH 3.4) for 1.5 h at 100°, cooled, centrifuged 1 h at 105,000 g, and the supernatant liquor was lyophilized and chromatographed on a 3.7 × 55 cm column packed with Sephadex G-50. The appropriate fractions were lyophilized. We obtained 125 mg of the polysaccharide, with $[\alpha]_D^{22}$ + 56.6° (C 1.0, water), and 140 mg of the oligosaccharide fraction.

N-Deacetylation of Polysaccharide. The polysaccharide (100 mg) was reduced with 20 mg of $NaBH_4$ in 3 ml of water for 2 h, acidified with AcOH, deionized by gel-chromatography on Sephadex G-25, and lyophilized. The reduced polysaccharide was dried in vacuo over P_2O_5 at 70° and then heated with 2 ml of anhydrous hydrazine and 100 mg of hydrazine sulfate in a sealed ampul for 20 h at 105°. The hydrazine was evaporated. The residue was dried in vacuo over H_2SO_4, chromatographed on a column packed with Sephadex G-50 (35 × 2.3 cm), and the fractions that escaped from the blank volume of the column were lyophilized. We obtained 72 mg of the N-deacetylated polysaccharide.

Acid Hydrolysis of N-Deacetylated Polysaccharide. Six 0.4-mg portions of the N-deacetylated polysaccharide were hydrolyzed with 0.5 ml of 2 N HCl at 100° in sealed ampuls. The hydrolyzates were cooled at definite time intervals, evaporated in vacuo at 20° over NaOH, and studied using an amino acid analyzer. Relative to the retention time of galactosamine, the retention time of disaccharide (II) was 0.62, and that of the modified acid component X was 1.17. The yields (%) of the hydrolysis products are given below, in which connection the amount of disaccharide (II), formed by hydrolysis in 3 h, was taken as 100%.

Time, h	1	2	3	4	6	8
Disaccharide (II)	63	84	100	98	98	95
Galactosamine	4	5	7	8	9	11

Isolation and Characteristics of Disaccharides (II) and (III). The N-deacetylated polysaccharide (30 mg) was hydrolyzed with 2 ml of 2 N HCl for 3 h at 100°, and the hydrolyzate was evaporated over NaOH at 20° and then chromatographed on a 11 × 0.6 cm column packed with cationite Dowex 50-W × 8, followed by initial elution with 0.025 M and then with 0.1 M pyridine – acetate buffer. The separation was checked by paper electrophoresis; disaccharide (II) has $M_{galactosamine}$ 0.65. The fractions that contained disaccharide (II) were acetylated with acetic anhydride in 90% methanol for 1 h, evaporated, and chromatographed on paper in system A, with elution of the zone with $R_{glucose}$ 0.58. Disaccharide (III) was obtained. Disaccharide (III) was incubated in 0.2 ml of 0.1 M phosphate – citrate buffer, pH 3.8, that contained β-hexosaminidase [11], and the cleavage was checked by PC in system A. After 5 h the disaccharide was completely cleaved into N-acetyl-galactosamine and galactose.

Periodate Oxidation. Here 10-mg portions of the starting and N-deacetylated polysaccharide were oxidized with 2 ml of 0.1 M $NaIO_4$ in the dark at 20°, after which 80 mg of $NaBH_4$ was added, and the mixture was kept for 2 h, acidified with AcOH, chromatographed on a column packed with Sephadex G-50, and the fractions that escaped from the blank volume of the column were lyophilized. GLC analysis revealed that the hydrolyzates of both of the oxidized polysaccharides contain galactosamine, galactose, and glucose in a 1:1:0.1 ratio. A 5-mg portion of the oxidized polysaccharide was hydrolyzed with 0.5 N HCl at 20° for 16 h and then lyophilized; gel-chromatography of the residue on Sephadex G-50 revealed that the polymeric fraction is completely absent.

Deamination of N-Deacetylated Polysaccharide. To 25 mg of the N-deacetylated polysaccharide in 1 ml of water were added 1.5 ml each of 5% $NaNO_2$ and 33% AcOH solutions. After 40 min the mixture was treated with cationite KU-2 (H^+ form), lyophilized, reduced with $NaBH_4$, treated with cationite KU-2 (H^+ form), evaporated; the H_3BO_3 was removed by evaporation with methanol, the residue was chromatographed on paper in system B, and the zones with $R_{lactose}$ 0.76 and 1.10 were eluted with water. We, respectively, obtained 5.2 mg of oligosaccharide (IV), with $[\alpha]_D^{20}$ + 73.9° (C 0.40, water), and 0.6 mg of an oligosaccharide

that gave glucose and galactose on hydrolysis. Oligosaccharide (IV) was acetylated with acetic anhydride in pyridine. Acetate (V) was obtained. Mass spectrum (m/e): 848 ($M-CH_3COOH$), 835 ($M-CH_2OAc$), 788 ($M-2CH_3COOH$), 619, 561, 331, 319, 273.

Methylation of Polysaccharide and Oligosaccharide (IV). The methylation was carried out in the presence of methylsulfinyl anion as described in [12]. In the case of the polysaccharide the mixture was diluted with water and dialyzed; in the case of oligosaccharide (IV) the methylated product was extracted with $CHCl_3$, and the extract was dried over Na_2SO_4 and evaporated. The methylated samples were hydrolyzed with 85% HCOOH for 2 h at 100°, and then with 0.3 N HCl for 16 h at 100°, evaporated, reduced with $NaBH_4$, worked up in the usual manner, and studied by the GLC – MS method as the partially methylated polyol acetates. The analysis results are given in Table 1. Mass spectrum of 1,4,6-tri-O-methyl-3-O-acetyl-2,5-anhydro-talitol (m/e): 203, 143, 129, 115, 111, 103, 101, 87, 85, 75, 71, 59, 55, 45, and 43. The mass spectra of the other partially methylated monosaccharides were interpreted on the basis of the data given in [13].

Determination of Configuration of Glycoside Linkages. Oligosaccharide (V) was oxidized with 20 mg of CrO_3 in 0.2 ml of glacial AcOH for 2 h at 50°, diluted with water, extracted with $CHCl_3$, and the extract was washed and evaporated. The residue was hydrolyzed with 2 N HCl and studied by GLC as the full polyol acetates. The galactose : glucose : 2,5-anhydro-talitol ratio was 1 : 1 : 0.15.

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CONCLUSIONS

Based on the data of selective cleavage, methylation, and oxidation with CrO_3 it was shown that the repeating unit of the polysaccharide chain of the liposaccharide from type 6 Sh. dysenteriae is the trisaccharide β-D-N-acetylgalactosaminyl-(1 – 3)-α-D-galactopyranosyl-(1 – 6)-α-D-glucopyranose, to the galactose residue of which is attached an unindentified acid component in the 4 position.

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