Carbohydrate Research, 40 (1975) 365–372

SELECTIVE CLEAVAGE OF GLYCOSIDIC LINKAGES: STUDIES WITH THE O-SPECIFIC POLYSACCHARIDE FROM *Shigella dysenteriae* TYPE 3

BORIS A. DMITRIEV, YURIY A. KNIREL, AND NIKOLAY K. KOCHETKOV
N. D. Zelinsky Institute of Organic Chemistry, Academy of Sciences of U.S.S.R., Moscow (U.S.S.R.)

(Received July 23rd, 1974; accepted for publication, August 16th, 1974)

ABSTRACT

Treatment of the O-specific polysaccharide from *Shigella dysenteriae* Type 3 with hydrazine in the presence of hydrazine sulphate resulted in quantitative *N*-deacetylation with the formation of a modified polysaccharide containing free amino groups. Oxidation of the modified polysaccharide with periodate did not destroy the 2-amino-2-deoxygalactose residues, thus indicating that they were substituted at position 3. Acid hydrolysis of the modified polysaccharide afforded 3-*O*-(2-amino-2-deoxy-β-D-galactopyranosyl)-D-galactose, which was identified as the *N*-acetyl derivative. Deamination of the modified polysaccharide with nitrous acid cleaved the 2-amino-2-deoxy-D-galactopyranosyl linkages to give a pentasaccharide as the major product, which appeared to be the modified chemical repeating unit of the O-specific polysaccharide.

INTRODUCTION

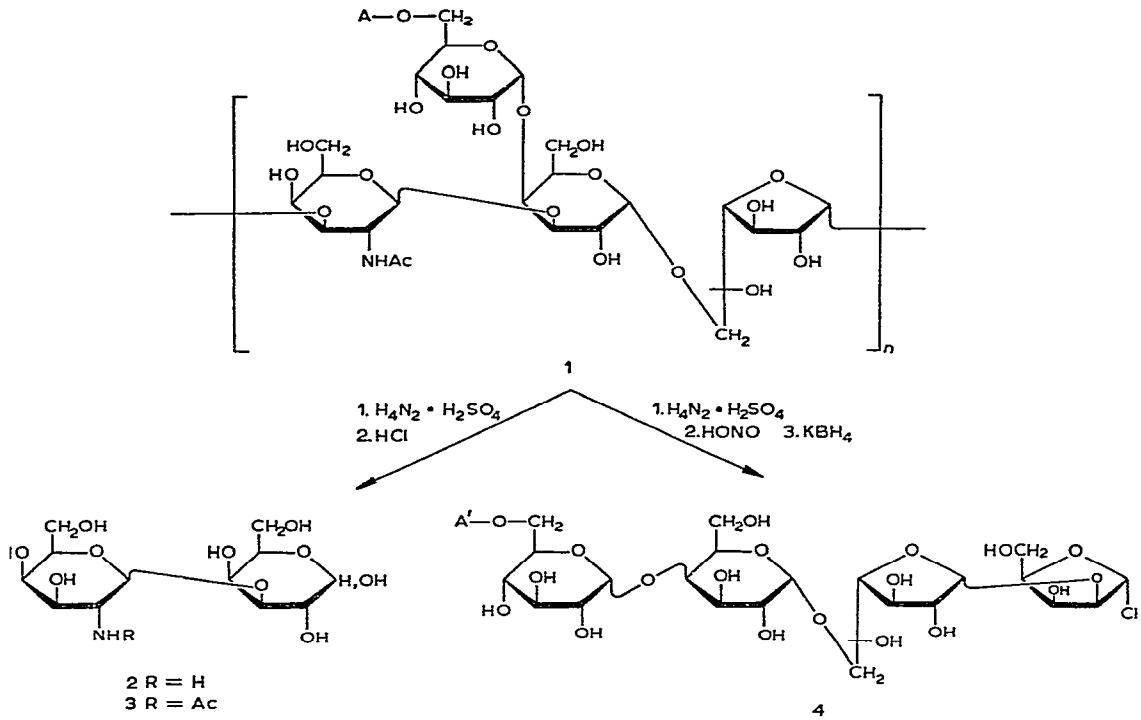
Despite numerous attempts, the successful selective fragmentation of hexosaminoglycans into oligosaccharides by sequential *N*-deacetylation and deamination reactions has only twice been achieved[1,2]. Incomplete *N*-deacetylation and destruction of the polysaccharides, as well as dependence of the extent of the deamination reaction on the position of substituents in hexosamine residues, are the main limitations of this approach.

We have defined[3–5] the optimal conditions for *N*-deacetylation, deamination, and acid hydrolysis with model biosides containing 2-acetamido-2-deoxyglucose residues substituted at positions 3, 4, or 6, and we now report the application of these procedures in a structural investigation of the O-specific polysaccharide chain from *Shigella dysenteriae* Type 3 lipopolysaccharide.

RESULTS AND DISCUSSION

The O-specific polysaccharide from *Shigella dysenteriae* Type 3 was obtained by mild acid hydrolysis of the lipopolysaccharide isolated from dry bacterial cells,

according to Westphal's procedure[6]. On the basis of methylation analysis, partial acid hydrolysis, and chromic anhydride-oxidation data, the polysaccharide was found[7] to be an acidic, branched hexosaminoglycan, the repeating unit of which was represented by the pentasaccharide (**1**) composed of 2-acetamido-2-deoxy-D-galactose, D-galactose, D-glucose, and an unidentified acidic monosaccharide (A) in the ratios 1:2:1:1.



After reduction with borohydride, the polysaccharide was *N*-deacetylated with anhydrous hydrazine–hydrazine sulphate. Such treatment did not cause any destruction of the polysaccharide, since the *N*-deacetylated material was quantitatively eluted from a column of Sephadex G-50 within the void volume. The modified polysaccharide had no i.r. absorption for amide groups and, on paper electrophoresis, it migrated towards the cathode as a sharp zone. The content of free amino groups in the modified polysaccharide, when determined by the trinitrobenzenesulphonate method[8], showed that *N*-deacetylation was complete. However, since the fate of the acidic component of the polysaccharide after the action of hydrazine was unknown (the formation of hydrazide was not excluded), the extent of *N*-deacetylation was also assessed on the basis of data for acid hydrolysis (see below).

The *N*-deacetylated polysaccharide was subjected, in sequence, to deamination with nitrous acid, acid hydrolysis, reduction with borohydride, and acetylation. The g.l.c. data for the resulting alditol acetates corresponded with those obtained

when the native polysaccharide was subjected in sequence to acid hydrolysis, deamination, reduction, and acetylation. Thus, the completeness of the *N*-deacetylation process was confirmed, as was the absence of changes in the quantitative ratio of neutral sugars and 2-amino-2-deoxygalactose in the polysaccharide after the hydrazinolysis.

Periodate oxidation before and after *N*-deacetylation may be used as a method for determining the positions of substituents in hexosamine units of polysaccharides[3]. The 2-amino-2-deoxygalactose residue in the *N*-deacetylated polysaccharide from *Shigella dysenteriae* Type 3 survived periodate oxidation and, thus, was substituted at position 3. This conclusion corresponded to that obtained on methylation analysis, which led to the identification of methyl 2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido)-α-D-galactopyranoside as the single component of a hexosamine fraction[7].

Further, since glycosides of amino sugars having free amino groups are relatively stable to acid hydrolysis, the *N*-deacetylated polysaccharide was treated with 2M hydrochloric acid at 100° with the aim of isolating a disaccharide having 2-amino-2-deoxy-D-galactose as the non-reducing moiety. The investigation of the hydrolysate, using an amino acid analyser, revealed the main product, which was identified as disaccharide **2**, together with a small proportion of free 2-amino-2-deoxygalactose. Hydrolysis for a short time led to incomplete release of the disaccharide, whereas prolonged hydrolysis caused partial decomposition. The optimal time of hydrolysis was 2 h, and a high yield of **2** was obtained and isolated by chromatography on a cation-exchange resin. The disaccharide appeared to be homogeneous on paper electrophoresis and analysis using an amino acid analyser. Deamination with nitrous acid cleaved **2** into galactose and 2,5-anhydrotalose (in the ratio 1:1) which were identified by g.l.c. as their alditol acetates. Thus, **2** was a 2-amino-2-deoxygalactosyl-galactose.

N-Acetylation of **2** gave a neutral compound (**3**) which was homogeneous in paper chromatography and gave a strong, positive reaction with the Morgan–Elson reagent[9]. Acid hydrolysis of **3** gave 2-amino-2-deoxygalactose and galactose in the ratio 1:1. Thus, **3** was 3-*O*-(2-acetamido-2-deoxy-D-galactopyranosyl)-D-galactopyranose. The presence of the (1→3) linkage between 2-amino-2-deoxygalactose and galactose residues in the polysaccharide was confirmed by methylation analysis of the fragment obtained from the *N*-deacetylated polysaccharide by deamination with nitrous acid, as described below. The β-D configuration of the glycosidic linkage in **3** was proved by its cleavage into galactose and 2-acetamido-2-deoxygalactose on incubation with β-D-hexosaminidase[10] from pig epididymis. This result was in agreement with the data on the chromic anhydride oxidation of the acetylated polysaccharide[7].

Deamination of 2-amino-2-deoxyglucosides and 2-amino-2-deoxygalactosides with nitrous acid cleaves the glycosidic bonds and yields 2,5-anhydromannose and 2,5-anhydrotalose, respectively[11]. The *N*-deacetylated polysaccharide from *Shigella dysenteriae* Type 3 was deaminated by sodium nitrite in dilute acetic acid under conditions which were found to be optimal for the cleavage of model biosides[3–5],

and the products were reduced with potassium borohydride. Subsequent chromatography on Sephadex G-50 and paper showed that the polysaccharide had been completely degraded into oligosaccharide fragments. On electrophoresis, the deamination product appeared to be a neutral material slightly contaminated with an acidic component which was removed by adsorption on an anion-exchange resin.

The neutral fraction was a mixture of two oligosaccharides with $R_{Lactose}$ values of 0.28 (major) and 0.4. The major component was isolated by preparative paper chromatography and identified as the pentasaccharide derivative **4**.

Acid hydrolysis of **4** gave 2,5-anhydrotalitol, glucose, and galactose in the ratios 1:1:2 (identified by g.l.c.). Methylation of **4** by the Hakomori procedure[12] followed by acid hydrolysis and identification, by g.l.c.–m.s., of the resulting partially methylated sugars gave the data in Fig. 1 and Table I. Thus, **4** was shown to be composed of 6-*O*-substituted galactofuranose, 4-*O*-substituted galactopyranose, 6-*O*-substituted glucopyranose, and 3-*O*-substituted 2,5-anhydrotalitol moieties. A g.l.c. peak for a derivative of the terminal acidic monosaccharide (A′) was not observed. Peaks of the minor components a–d (Fig. 1*a*), as was shown earlier[7], were due to monosaccharides originating from the core oligosaccharide to which the O-specific polysaccharide chain is known to be attached in the lipopolysaccharide, and were not considered. The low yields of 2,3,5-tri-*O*-methylgalactofuranose (Fig. 1, peak D) and tri-*O*-methyl-2,5-anhydrotalitol (peak A) were caused by decomposition of the former under the conditions of acid hydrolysis[7] and by the high volatility of the latter. The absence of any fully methylated monosaccharide indicated that the non-reducing terminus of **4** was a neutral derivative of the acidic monosaccharide observed in the native polysaccharide (peak F).

The methylation-analysis data presented above, together with those for the monosaccharide sequence in *Shigella dysenteriae* Type 3 polysaccharide[7], establish the structure of oligosaccharide **4**, which represents the modified chemical repeating-unit of the specific polysaccharide. Comparison of the results of methylation analysis for the polysaccharide and the pentasaccharide **4** (Table I and Fig. 1) shows that 2,3,6-tri-*O*-methylgalactopyranose (peak B) appeared in the oligosaccharide instead of the 2,6-di-*O*-methyl derivative (peak E). These data prove that the 2-amino-2-deoxygalactose residue in the polysaccharide is attached to a di-*O*-substituted galactopyranose residue at position 3 and are in agreement with those for the identification of disaccharide **3** obtained by selective hydrolysis of the *N*-deacetylated polysaccharide.

The results on the selective cleavage of glycosidic linkages in *Shigella dysenteriae* Type 3 polysaccharide, presented herein, establish that the polysaccharide is built up of regularly repeating pentasaccharide units.

EXPERIMENTAL

General. — Paper chromatography (p.c.) was effected by the descending method on FN-11 paper, using *A* 1-butanol–pyridine–water (6:4:3) and *B* ethyl

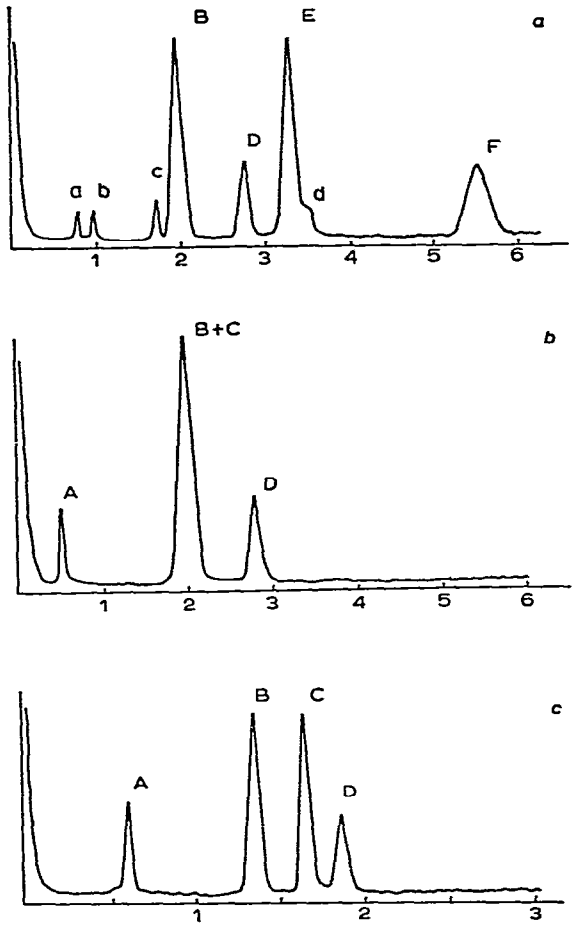


Fig. 1. Gas chromatograms of partially methylated alditol acetates obtained from methylated polysaccharide (*a*) and methylated oligosaccharide 4 (*b* and *c*); *a* and *b*, column *A*, *c*, column *B*.

TABLE I

G.L.C.–M.S. ANALYSIS OF METHYLATED POLYSACCHARIDE (PS) AND OLIGOSACCHARIDE 4

Peaks	*Identity*	T_R[a]		*Molar ratios*	
		Column A	*Column* B	*PS*	4
A	2,5-Anhydro-1,4,6-tri-*O*-methyltalitol	0.5	0.6	—	0.2
B	2,3,6-Tri-*O*-methylgalactitol	1.96	1.38	—	1.0
C	2,3,4-Tri-*O*-methylglucitol	2.00	1.54	1.0	1.0
D	2,3,5-Tri-*O*-methylgalactitol	2.76	1.74	0.4	0.4
E	2,6-Di-*O*-methylgalacitol	3.14	1.82	1.0	—
F	Derivative of unidentified, acidic sugar	5.48	2.12	1.0	—

[a] T_R relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol.

acetate–acetic acid–formic acid–water (18:3:1:4). Electrophoresis was carried out on FN-11 paper in 25mM pyridine acetate buffer (pH 4.5) at a gradient of 28 volts/cm for 90 min. Alkaline silver nitrate was used for detection of materials on paper; polysaccharide was detected after exposure of the paper to steam. Gel filtration was performed on Sephadex G-50 (column, 55 × 3.7 cm) and G-25 (column, 35 × 2.3 cm) in pyridine acetate buffer (10 ml of acetic acid, 4 ml of pyridine, and 1 litre of water); elution was monitored by the phenol–sulphuric acid procedure[14]. Analysis of amino sugars was carried out at 75°, using an amino acid analyser BC-200 (Biocal-LKB) fitted with a column (27 × 0.9 cm) of Chromex UA-8 resin and elution with 0.35M sodium citrate buffer (pH 5.28). G.l.c. was carried out on a Pye Unicam Series 104 (Model 64) instrument with a dual flame-ionization detector, including an Autolab digital integrator Vidar-6300, a glass column (90 × 0.4 cm) packed with 3% ECNSS-M on Gaschrom Q (100–120 mesh, column *A*), and a stainless-steel column (1.5 m × 0.4 cm) packed with 3% SE-30 on Diatomite CQ (80–100 mesh, column *B*). The nitrogen flow-rate was 40 ml/min. G.l.c.–m.s. was performed on a Varian instrument (Gnom Mat 111), with the use of columns described above. Solvents were evaporated under diminished pressure with a bath temperature below 40°.

N-*Deacetylation of the polysaccharide.* — A portion (70 mg) of the O-specific polysaccharide, isolated and characterised as described recently[7], was reduced at room temperature with potassium borohydride (20 mg) in water (3 ml) for 2 h. The excess of reagent was destroyed by addition of acetic acid, and the solution was desalted by passing through a column of Sephadex G-25 and freeze-dried. The reduced polysaccharide was dried *in vacuo* over P_2O_5 at 70° and then heated with anhydrous hydrazine (2 ml) containing hydrazine sulphate (100 mg) in a sealed tube for 10 h at 105°. Hydrazine was removed by evaporation, and the residue was dried over conc. sulphuric acid *in vacuo* and then purified by gel filtration on a column of Sephadex G-50. The elution curve contained one peak eluted within the void volume of the column. Appropriate fractions were combined and freeze-dried to give the *N*-deacetylated polysaccharide (62 mg). The material obtained was homogeneous and, on paper electrophoresis, moved towards the cathode as a sharp zone.

Periodate oxidation of the N-*deacetylated polysaccharide.* — A solution of the *N*-deacetylated polysaccharide (5 mg) in 0.1M sodium periodate (2 ml) was kept in the dark at room temperature for 48 h, then reduced with potassium borohydride (80 mg) for 2 h, and acidified with acetic acid. The solution was passed through a column of Sephadex G-25, fractions eluted within the void volume were concentrated, and the residue was hydrolyzed with 2M hydrochloric acid (2.5 ml) for 4 h at 100°. After cooling, the solution was evaporated and a solution of the residue in water (0.3 ml) was treated in succession with 33% acetic acid (0.5 ml) and 5% sodium nitrite (0.5 ml). The mixture was kept for 40 min at 20°, then treated with KU-2(H^+) resin, and freeze-dried. The residue was conventionally reduced with potassium borohydride, and the resulting alditols were analysed in the form of their acetates by g.l.c.–m.s. on column *A* at 190°; glucitol, galactitol, and 1,5-anhydrotalitol in the ratios 0.1:1:1 were detected.

Selective cleavage of the N-*deacetylated polysaccharide with acid.* — Seven portions (0.5 mg) of *N*-deacetylated polysaccharide were hydrolysed severally in sealed tubes with 2M hydrochloric acid (0.5 ml) at 100°. After cooling, the solutions were evaporated over sodium hydroxide in a vacuum desiccator and then investigated by using an amino-acid analyser. The results were as follows:

Time (h)	0.5	1	1.5	2	2.5	3	4
Disaccharide **2**[a] (%)	74	83	92	100	97	87	81
2-Amino-2-deoxygalactose (%)	2	4	5	5	7	10	14

[a]Elution time of **2** is 0.62 relative to that of 2-amino-2-deoxygalactose, the yield of **2** after hydrolysis for 2 h being taken as 100%.

In a separate experiment, *N*-deacetylated polysaccharide (25 mg) was hydrolysed with 2M hydrochloric acid (2 ml) for 2 h, and the product mixture was fractionated on a column (11 × 0.6 cm) of Dowex-50W x8(H^+) resin by elution with a linear gradient of pyridine acetate buffer 0.025–0.1M, the separation being monitored by electrophoresis. Fractions containing **2** (mobility 0.65 relative to that of 2-amino-2-deoxygalactose) were combined and freeze-dried.

A portion of the residue was deaminated, reduced, and investigated by g.l.c., as described above, to give 2,5-anhydrotalitol and galactitol in the ratio 1:1.

A second portion of **2** was *N*-acetylated with acetic anhydride in the presence of Amberlite IRA-401(CO_3^{2-}) resin in 10% aqueous methanol for 2 h to give **3**, which reacted positively with Morgan–Elson reagent[9] after p.c.: R_{Glc} 0.58 (solvent *A*) and 0.81 (solvent *B*).

A solution of **3** (0.5 mg) in 0.1M phosphate–citrate buffer (pH 3.8, 0.2 ml) was incubated with a β-D-hexosaminidase preparation[10] from pig epididymis, and enzymic hydrolysis was monitored by p.c. in (solvent *A*). Disaccharide **3** was completely cleaved into galactose and 2-acetamido-2-deoxygalactose within 5 h.

Selective cleavage of the N-*deacetylated polysaccharide by deamination.* — A solution of *N*-deacetylated polysaccharide (30 mg) in water (1 ml) was treated in succession with 33% acetic acid (1.5 ml) and 5% sodium nitrite (1.5 ml) and then kept for 40 min at room temperature. The mixture was passed through a column of KU-2(H^+) resin and then freeze-dried, and the residue was reduced with potassium borohydride. After routine treatment, the solution was passed through Amberlite IRA-401(CO_3^{2-}) resin to give a neutral fraction, which was separated by preparative p.c. (solvent *B*) into two oligosaccharides having mobilities $R_{Lactose}$ 0.28 (major, **4**) and 0.40.

A portion of **4** was hydrolysed with 2M hydrochloric acid for 4 h at 100° to give 2,5-anhydrotalitol, galactose, and glucose in the ratios 1:2:1, identified by g.l.c.

A second portion of **4** was methylated by the Hakomori procedure[12], and the mixture was diluted with water and extracted with chloroform. The organic layer was washed with water, dried (Na_2SO_4), and concentrated. The residue was treated in succession with 85% formic acid for 2 h at 100° and 0.3M hydrochloric acid for

16 h at 100°, and then concentrated to dryness, and the residue was reduced with sodium borodeuteride. The resulting alditols were investigated in the form of their acetates by g.l.c.–m.s. on column *A* at 160° and column *B* at 180° (see Fig. 1). The results of methylation analysis are presented in Table I.

ACKNOWLEDGMENTS

The authors are greatly indebted to Drs. I. L. Hofman for cultivation of microbial cells, and to G. V. Vikha for the gift of a β-D-hexosaminidase preparation.

REFERENCES

1 M. ISEMURA AND K. SCHMID, *Biochem. J.*, 124 (1971) 591.
2 S. HASE AND Y. MATSUSHIMA, *J. Biochem. (Tokyo)*, 72 (1972) 1179.
3 B. A. DMITRIEV, YU. A. KNIREL, AND N. K. KOCHETKOV, *Carbohyd. Res.*, 29 (1973) 451.
4 B. A. DMITRIEV, YU. A. KNIREL, AND N. K. KOCHETKOV, *Izv. Akad. Nauk SSSR, Ser. Khim.*, (1974) 411.
5 B. A. DMITRIEV, YU. A. KNIREL, AND N. K. KOCHETKOV, *Carbohyd. Res.*, 30 (1973) 45.
6 O. WESTPHAL AND K. JANN, *Methods Carbohyd. Chem.*, 5 (1965) 83.
7 B. A. DMITRIEV, L. V. BACKINOWSKY, V. L. LVOV, N. K. KOCHETKOV, AND I. L. HOFMAN, *Eur. J. Biochem.*, (1975) in press.
8 G. T. GALAMBOS AND R. SHAPIRO, *Anal. Biochem.*, 15 (1966) 334.
9 R. KUHN, A. GAUHE, AND H. H. BAER, *Chem. Ber.*, 89 (1956) 1027.
10 G. V. VIKHA, E. D. KAVERZNEVA, AND A. YA. KHORLIN, *Biokhimia*, 36 (1971) 33.
11 D. HORTON, in R. W. JEANLOZ (Ed.), *Amino Sugars*, Academic Press, New York, 1969, p. 128.
12 H. E. CONRAD, *Methods Carbohyd. Chem.*, 6 (1972) 361.
13 H. BJÖRNDAL, B. LINDBERG, AND S. SVENSSON, *Carbohyd. Res.*, 5 (1967) 433.
14 M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH, *Anal. Chem.*, 28 (1956) 350.