Carbohydrate Research, 29 (1973) 451–457

SELECTIVE CLEAVAGE OF GLYCOSIDIC LINKAGES

STUDIES WITH THE MODEL COMPOUND BENZYL 2-ACETAMIDO-2-DEOXY-3-*O*-β-D-GALACTOPYRANOSYL-α-D-GLUCOPYRANOSIDE

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

(Received September 19th, 1972; accepted for publication in revised form, February 12th, 1973)

ABSTRACT

Benzyl 2-acetamido-2-deoxy-3-*O*-β-D-galactopyranosyl-α-D-glucopyranoside (**1**) was chosen as a model bioside to develop a standard procedure for the selective cleavage of glycosidic linkages in polysaccharides containing 2-amino-2-deoxyhexose residues. Treatment of **1** with hydrazine in the presence of hydrazine sulphate resulted in quantitative *N*-deacetylation with the formation of benzyl 2-amino-2-deoxy-3-*O*-β-D-galactopyranosyl-α-D-glucopyranoside (**2**). The galactosyl glycosidic linkage in **2** could be selectively cleaved by acid hydrolysis. Oxidation of **2** with periodate destroyed the galactose residue. Treatment of **2** with nitrous acid cleaved the 2-amino-2-deoxy-D-glucosyl linkage to give 2,5-anhydro-3-*O*-β-D-galactopyranosyl-D-mannose (**3**) and benzyl alcohol.

INTRODUCTION

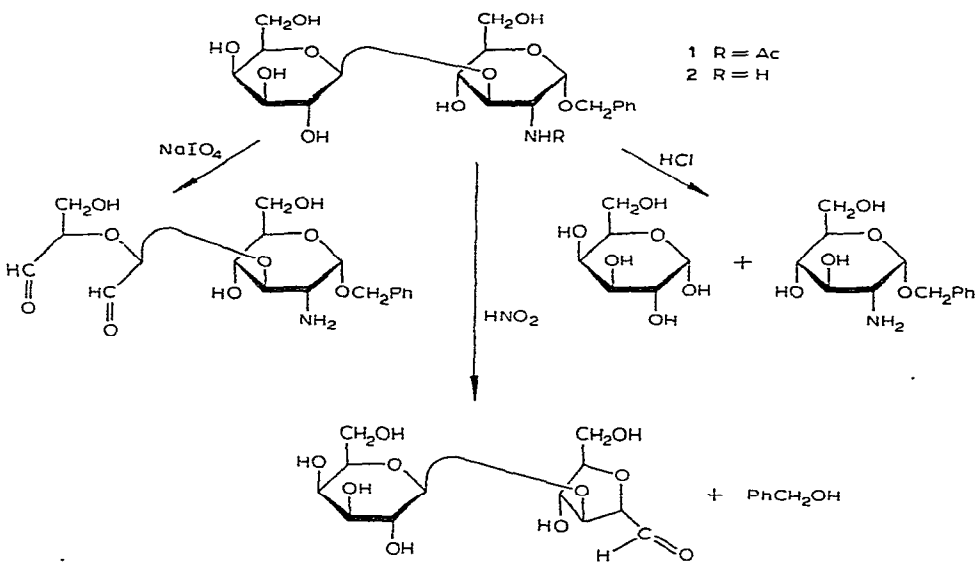
The *O*-specific polysaccharide chains of bacterial cell-wall lipopolysaccharides from *Shigella flexneri*[1] and some *Salmonella* species[2] contain 2-acetamido-2-deoxyhexose residues as constituents of the repeating oligosaccharide units. Structural investigation of such polysaccharides based upon partial hydrolysis, followed by the isolation and identification of 2-amino-2-deoxyhexose(hexosamine)-containing oligosaccharides[3], is rather tedious. Selective cleavage of 2-amino-2-deoxyhexosyl linkages, after *N*-deacetylation and subsequent deamination, seems to be an attractive, alternative approach. However, the procedure for the selective fragmentation of mucopolysaccharides[4] based on this principle has not been generally used. Incomplete *N*-deacetylation and destruction of the polysaccharide chain have been noted[5]. Moreover, deamination may be incomplete, and the extent of the reaction appears to depend on the position of substituents in the hexosamine molecule[6]. The aim of our investigation was to elaborate conditions for the selective cleavage of the glycosidic linkages in the model bioside **1** by effecting, in sequence, *N*-deacetylation, deamination, acid hydrolysis, and periodate oxidation.

RESULTS AND DISCUSSION

The known methods of *N*-deacetylation of hexosaminides comprise alkaline hydrolysis[7,8], hydrazinolysis[9,10], treatment with triethyloxonium fluoroborate[11], and treatment with phosphorus pentasulphide[12]. The last two procedures are applicable to *O*-substituted *N*-acetylhexosamine derivatives, whereas the first two methods are suitable for application to **1** since unsubstituted hydroxyl groups do not prevent the action of the reagents. Recently, treatment with 2.5M sodium hydroxide was applied for the *N*-deacetylation of the α_1-acid glycoprotein[8] of human plasma. The reaction proceeded to an extent of 60% and was not accompanied by destruction of the polysaccharide chain. *N*-Deacetylation of **1** with 2.5M sodium hydroxide at 100° yielded 65% of **2**. Significant destruction of **2** did not occur even after prolonged heating. The bioside **2** gave a crystalline hepta-acetate which was identical with the authentic compound described by Flowers and Jeanloz[13].

Hydrazinolysis of **1** was carried out by using anhydrous hydrazine, hydrazine hydrate[9], or hydrazine in the presence of hydrazine sulphate[10]. The bioside **2** was obtained in yields of 66% and 42% upon treatment of **1** with anhydrous hydrazine and hydrazine hydrate, respectively; no decomposition of the biosides was observed. The incomplete *N*-deacetylation of **1** conforms to the observation that 3-*O*-substituted 2-acetamido-2-deoxyhexosides are difficult to *N*-deacetylate[9]. However, hydrazinolysis of **1** proceeded smoothly in the presence of catalytic amounts of hydrazine sulphate to give **2** in quantitative yield. Thus, the reagent proposed by Yosizawa *et al.*[10] apparently is not affected by substitution in the hexosamine residue.

The biosides **1** and **2** each consumed 2 moles of periodate with concomitant



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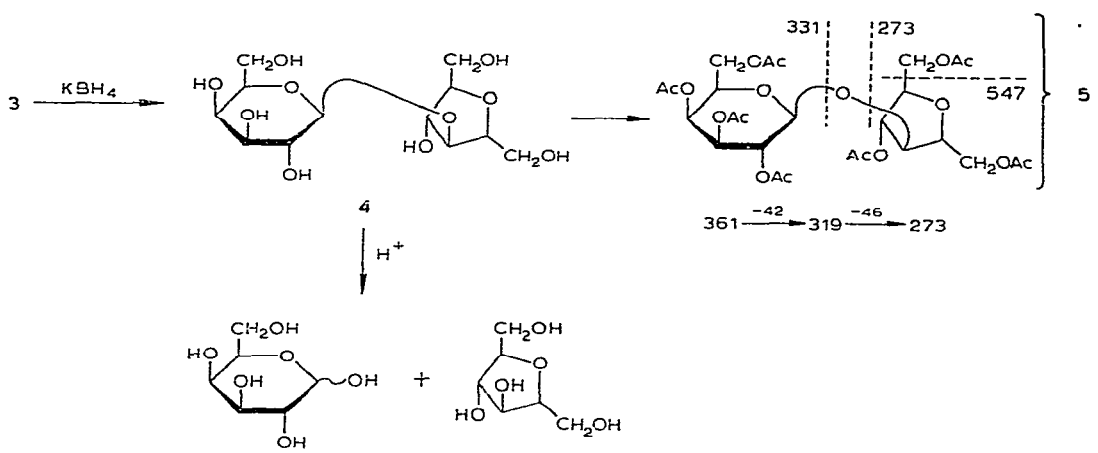
destruction of the galactose moiety. Periodate oxidation before and after *N*-deacetylation is useful for determining the position of substituents in hexosamine units in mucopolysaccharides. 3-*O*-Substituted hexosaminides survive the periodate treatment both before and after *N*-deacetylation. 4-*O*-Substituted hexosaminides will be resistant only in the *N*-acetylated form, whereas the 6-*O*-substituted derivatives will be destroyed both before and after *N*-deacetylation. Recently, this approach was used for the isolation of oligosaccharides containing 3-*O*-substituted hexosamine residues[14], and in a structural investigation of the K-antigen isolated from *Escherichia coli*[15].

Acid hydrolysis of **2** with M and 2M hydrochloric acid at 100° for 2 and 1 h, respectively, resulted in the quantitative formation of galactose and benzyl 2-amino-2-deoxy-D-glucopyranoside. The appearance of 2-amino-2-deoxy-D-glucose in the hydrolysate was observed after prolonged heating. These conditions of acid hydrolysis may be recommended for the selective cleavage of *N*-deacetylated mucopolysaccharides. Similar treatment (0.5M sulphuric acid, 5 h, 100°) has been reported[16] for the fragmentation of *N*-deacetylated blood-group A substance into disaccharides containing terminal, non-reducing 2-amino-2-deoxy-D-glucose residues.

Deamination of 2-amino-2-deoxy-D-glucosides with nitrous acid results in cleavage of the glycosidic bond with the formation of 2,5-anhydro-D-mannose or its derivatives[4]. Deamination[17] of **2** yielded ~70% of 2,5-anhydro-3-*O*-β-D-galactopyranosyl-D-mannose (**3**) and benzyl alcohol. The presence of an additional, unidentified compound in the product mixture after deamination was demonstrated by borohydride treatment of the mixture followed by acid hydrolysis and g.l.c. Deamination of **2** under the conditions recommended by Dische[18] for the quantitative determination of hexosamines gave **3** and benzyl alcohol (identified as the acetate) in quantitative yield. Reduction of **3** with potassium borohydride gave 2,5-anhydro-3-*O*-β-D-galactopyranosyl-D-mannitol (**4**) which was isolated as the hepta-acetate. The structure of the hepta-acetate was proved by mass spectrometry.



The principal fragmentation pathways shown in **5** need no comment, except for the short series of peaks at *m/e* 361→319→273. The peak at *m/e* 273 corresponds to the tri-*O*-acetyl-2,5-anhydro-D-mannitol fragment, and the precursor of this ion has *m/e* 361 which is generated by cleavage of the C-1–C-2 and C-1–O-1 bonds in the galactose residue with simultaneous migration of OAc-1, as described in the fragmentation of acetylated glycosides of serine and threonine[19]. The ion at *m/e* 361 successively loses ketene (−42) and formic acid (−46) to give an ion at *m/e* 273.

The reduction **3**→**4** effected with potassium borohydride was accompanied by β-elimination of the galactose residue. This type of reaction is well known for (1→3)-linked disaccharides in alkaline media. Galactitol was identified (g.l.c., t.l.c.) among the reaction products, and ~20% of **3** underwent a β-elimination reaction.

EXPERIMENTAL

General. — T.l.c. was performed on Silica Gel KSK, with detection by heating with conc. sulphuric acid or orcinol. Amines were detected with ninhydrin. Ascending paper chromatography (p.c.) was performed on Whatman No. 1 paper, using 1-butanol–ethanol–water (3:2:2) and detection with alkaline silver nitrate and ninhydrin reagents. G.l.c. was effected with an LCM-8-MD instrument fitted with a flame-ionization detector, a glass-spiral column (1 m × 3 mm) packed with 3% poly(neopentylglycol succinate) on Chromosorb W (60–80 mesh), and a nitrogen flow-rate of 50 ml/min. Optical rotations were determined with a Perkin–Elmer Model 141 polarimeter. Mass spectra were recorded with a Varian CH-6 instrument at an inlet temperature of 80°, and an ionizing potential of 70 eV. Melting points were determined with a Koffler apparatus. Evaporations were conducted *in vacuo* with a bath temperature below 40°.

Alkaline hydrolysis of **1**. — Samples (12 mg) of **1**, prepared as described by Flowers and Jeanloz[13], were dissolved in 2.5M sodium hydroxide (0.5 ml), and the solutions were heated for 2.5 and 5 h at 90° in sealed tubes. After cooling, the solutions were passed through a column of Dowex-50 x4(H^+) resin (1 ml) and the column was washed with distilled water to give 15 ml of eluate. Elution was then continued with 2M ammonium hydroxide (15 ml). The eluate was evaporated, the residue was dissolved in water (15 ml), the amount of unchanged **1** in the neutral eluate was determined by the phenol–sulphuric acid procedure[20], and the amounts of **2** in the ammonia eluate were determined by the trinitrobenzenesulphonate[21] and phenol–sulphuric acid procedures. The yields of **2** were 58–60% and 63–65% after heating for 2.5 and 5 h, respectively. Evaporation of the neutral eluate gave 35–40% of **1**. The ammonia eluate was evaporated, and the residue was dried over conc. sulphuric acid to give **2** as a white solid (60–65%), $[\alpha]_D^{20}$ +83° (*c* 1, water). The material was homogeneous on p.c. and t.l.c. (1-butanol–water, 86:14).

A solution of **2** (5 mg) in pyridine (0.5 ml) was treated with acetic anhydride (0.5 ml) for 16 h at room temperature. The mixture was evaporated, and the residue was dried by repeated distillation of toluene therefrom. Crystallization of the residue from acetone–ether gave benzyl 2-acetamido-2-deoxy-3-*O*-β-D-galactopyranosyl-α-

D-glucopyranoside hexa-acetate (5 mg), m.p. 173–174°, $[\alpha]_D^{20}$ +51° (*c* 0.5, chloroform); lit.[13] m.p. 175–175°, $[\alpha]_D$ +45°.

Hydrazinolysis of **1**. — Three portions (15 mg) of **1** were heated severally in sealed tubes with hydrazine (0.5 ml), hydrazine hydrate (0.5 ml), and hydrazine (0.5 ml) in the presence of hydrazine sulphate (5 mg) at 105° for 5 and 10 h. After cooling, hydrazine was removed by distillation of toluene thrice from the residue which was then dried over conc. sulphuric acid. Solutions of the residues in water (1 ml) were applied to the top of columns of Dowex-50 x4(H^+) resin (1 ml), and eluted as described above to give neutral and alkaline fractions. The latter fractions were evaporated, and the residues were thoroughly extracted with ether to remove acetylhydrazine prior to analysis. The following results were obtained.

Reagent	*Reaction time (h)*	**1** *recovered* (%)	**2** *formed* (%)	*Total recovery* (%)
N_2H_4	5	55	41	96
	10	37	65	103
$N_2H_4 \cdot H_2O$	5	60	35	95
	10	55	42	97
$N_2H_4 \cdot H_2SO_4$	5	28	69	97
	10	4	98	102

Benzyl 2-amino-2-deoxy-α-D-glucopyranoside. — Benzyl 2-acetamido-2-deoxy-α-D-glucopyranoside[22] (20 mg) was heated with the hydrazine–hydrazine sulphate reagent (1 ml) at 105° for 10 h. The mixture was treated as described above, and the crude product was eluted from silica gel (5 g) with 1-butanol–water (86:14), to give the title compound (12 mg) as an amorphous, white solid, $[\alpha]_D^{20}$ +112° (*c* 0.6, water), which was homogeneous on t.l.c. (1-butanol–water). Treatment of the product with acetic anhydride (0.5 ml) in methyl alcohol (2 ml) regenerated the parent compound[22], m.p. 178–180°, $[\alpha]_D^{20}$ +155° (*c* 2, water).

Periodate oxidation of **2**. — A solution of **2** (5 mg) in 15mM sodium periodate (3 ml) was kept in the dark at room temperature, and the consumption of periodate was monitored spectrophotometrically at 310 nm. The results were as follows.

Time (h)	0.25	0.5	1	2	4	24
Oxidant consumed (mol.)	1.02	1.37	1.60	1.72	1.78	1.94

The final solution was freeze-dried, and the residue was extracted with pyridine (1 ml) to remove inorganic salts. The extract was evaporated, and the residue was dissolved in 2M hydrochloric acid (2 ml) and heated at 100° for 1.5 h. Water (2 ml) was added to the cooled hydrolysate, and the solution was neutralized with silver carbonate

and centrifuged. The supernatant was passed through a column of Dowex-50 x4(H^+) resin (1 ml) and eluted as described above to give neutral and alkaline eluates. Each eluate was evaporated, and the products were identified by t.l.c. The neutral eluate contained no galactose, and the alkaline eluate contained only benzyl 2-amino-2-deoxy-α-D-glucopyranoside.

Hydrolysis of **2** *with acid.* — (*a*) Samples (15 mg) of **2** were dissolved in M and 2M hydrochloric acid (2.5 ml), respectively. Aliquots (0.5 ml) were heated in sealed tubes at 100°, then cooled, and neutralized with M sodium hydroxide, and the volume of each solution was adjusted to 2 ml. 2-Amino-2-deoxy-D-glucose was determined by the modified Elson–Morgan procedure[23] and galactose by the Park–Johnson method[24]. The following results were obtained.

Time (*h*)	M *HCl*		2M *HCl*	
	Galactose (%)	*Glucosamine* (%)	*Galactose* (%)	*Glucosamine* (%)
0.25	33	0	67	0
0.5	55	0	87	0
0.75	70	0	95	0
1	79	0	98	1
2	97	0.5	—	—

(*b*) A sample (2 mg) of **2** was dissolved in M hydrochloric acid (1 ml) and heated at 100° for 2 h. After cooling, the hydrolysate was evaporated, and a solution of the residue in water (1 ml) was passed over Amberlite IRA-400(CO_3^{2-}) resin. The eluate was concentrated, and the products were identified by p.c. and t.l.c. as galactose and benzyl 2-amino-2-deoxy-α-D-glucopyranoside.

Cleavage of **2** *by nitrous acid.* — (*a*) Deamination by nitrous acid was carried out essentially as described by Hase and Matsushima[17]. A solution of **2** (6 mg) in water (2 ml) was mixed with 0.45M sodium acetate buffer (pH 3.5) and 5% aqueous sodium nitrite (2 ml). The mixture was kept at room temperature, and, at intervals, aliquots (0.5 ml) were diluted with 12.5% aqueous ammonium sulphamate (0.5 ml) and assayed by the indole–hydrochloric acid procedure[18]. The results are recorded below, the yield of **3** obtained by deamination using Dische's conditions[18] being taken as 100%.

Time (h)	0.5	1	2	4	8
Yield of **3** (%)	41	56	63	68	70.5

(*b*) Solution of **2** (12 mg) in water (4 ml) was treated in succession with 33% acetic acid (4 ml) and 5% aqueous sodium nitrite (4 ml). The mixture was stirred for 30 min, then neutralized with Dowex-2(CO_3^{2-}) resin, and filtered. The filtrate was

extracted with ether (5 × 3 ml), and the extract was dried (Na_2SO_4) and concentrated to a small volume. Benzyl alcohol was identified in the residue by g.l.c. at 120°, directly and as benzyl acetate after acetylation. The aqueous solution remaining after ether extraction was treated with an excess of potassium borohydride overnight at room temperature. The mixture was neutralized to pH 3 with KU-2(H^+) resin and evaporated. Boric acid was removed from the residue by several distillations of methanol therefrom under reduced pressure. The residue was dissolved in acetic anhydride–pyridine (1:1, 2 ml), the solution was kept overnight and then evaporated *in vacuo*, and the residue was dried by distillation of toluene therefrom under diminished pressure until the odour of pyridine could not be detected. The material was separated by p.l.c., using chloroform–acetone (19:1), to give hexa-*O*-acetyl-galactitol (~1 mg) and the hepta-acetate of **4** (5 mg), as an amorphous solid, $[\alpha]_D^{20}$ +90.2° (*c* 0.35, chloroform).

The hepta-acetate was deacetylated, using methanolic sodium methoxide, and then hydrolysed with M sulphuric acid (2 ml) at 100° for 4 h. After neutralisation, galactose and 2,5-anhydro-D-mannitol were identified by g.l.c. according to a procedure described elsewhere[25].

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