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SYNTHESIS OF A DI- AND A TRI-SACCHARIDE RELATED TO THE K-ANTIGEN OF *Klebsiella* TYPE 10 AND A STUDY OF THEIR INHIBITION IN THE PRECIPITIN REACTION

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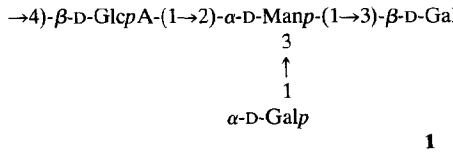
ABSTRACT

Syntheses of methyl 3-*O*-α-D-galactopyranosyl-α-D-mannopyranoside (**10**) and methyl 3-*O*-α-D-galactopyranosyl-2-*O*-(β-D-glucopyranosyluronic acid)-α-D-mannopyranoside (**11**) in good yield are described. Both **10** and **11** significantly inhibit antigen–antibody precipitation in the *Klebsiella* Type 10 immune system. The results provide more evidence for the structure (**1**) of the antigen from *Klebsiella* K-10 and its immunodominant grouping.

INTRODUCTION

We have reported[1] the structure **1** for the repeating unit of the capsular polysaccharide from *Klebsiella* Type 10. In the structure **2**, reported[2] later, D-glucose and D-glucuronic acid residues were included in the side chain and the Gal→Man linkage was designated as β. Although the results of acid hydrolysis and methylation analysis accorded with structure **2**, those of base-catalysed degradation, graded hydrolysis, and periodate-oxidation study did not.



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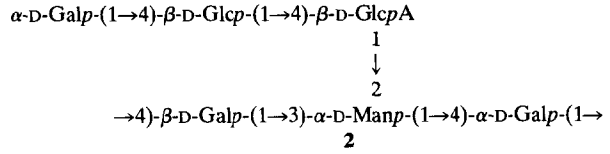

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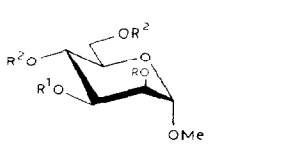
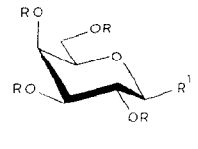
Therefore, we have sought immunochemical evidence for the repeating unit of the K-10 polysaccharide and also to identify the immunodominant group. The syntheses of methyl 3-*O*-α-D-galactopyranosyl-α-D-mannopyranoside (**10**) and methyl 3-*O*-α-D-galactopyranosyl-2-*O*-(β-D-glucopyranosyluronic acid)-α-D-mannopyranoside (**11**), which are parts of the repeating unit of K-10 antigen, were therefore undertaken in order to ascertain their effect in the *Klebsiella* Type 10 immune system.

RESULTS AND DISCUSSION

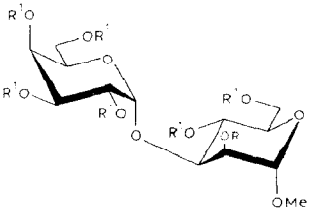
Methyl 4,6-di-*O*-benzyl-α-D-mannopyranoside[4] (**3**), prepared from methyl α-D-mannopyranoside[3], was partially allylated by the phase-transfer method[5,6] using allyl bromide and tetrabutylammonium bromide to give the 2-*O*-allyl derivative **4**. Methylation[7] of **4** followed by deallylation and debenzylation gave methyl 3-*O*-methyl-α-D-mannoside, which was identified by g.l.c. after conversion into the alditol acetate.



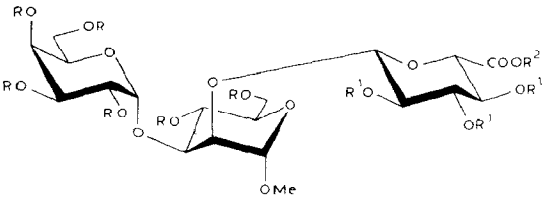
3 R = R¹ = H, R² = Bzl
4 R = Allyl, R¹ = H, R² = Bzl



5 R = Bzl, R¹ = SEt
6 R = Bzl, R¹ = Br



7 R = Allyl, R' = Bzl
8 R = H, R' = Bzl
10 R = R' = H

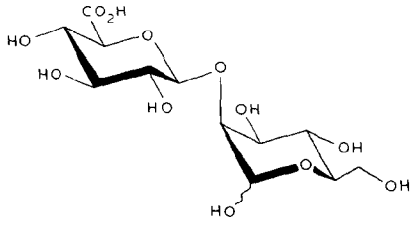


9 R = Bzl, R' = Ac, R² = Me
11 R = R' = R² = H

Condensation of **4** with 2,3,4,6-tetra-*O*-benzyl-α-D-galactopyranosyl bromide (**6**), obtained by treatment of ethyl 2,3,4,6-tetra-*O*-benzyl-1-thio-β-D-galactopyranoside (**5**) with bromine, in the presence of tetraethylammonium bromide gave methyl 2-*O*-allyl-4,6-di-*O*-benzyl-3-*O*-(2,3,4,6-tetra-*O*-benzyl-α-D-galactopyranosyl)-α-D-mannopyranoside (**7**, ~65%). The pure compound had $[\alpha]_D$ +61°, indicating the new linkage to be α (*cf.* refs. 8 and 9). Treatment of **7** with potassium *tert*-butoxide followed by mercury(II) oxide and mercury(II) chloride[10] gave methyl 4,6-di-*O*-benzyl-3-*O*-(2,3,4,6-tetra-*O*-benzyl-α-D-galactopyranosyl-α-D-mannopyranoside (**8**). Condensation of **8** with methyl (2,3,4-tri-*O*-acetyl-α-D-glucopyranosyl

bromide)uronate[11] in the presence of mercury(II) cyanide under nitrogen gave methyl 4,6-di-*O*-benzyl-2-*O*-(methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosyluronate)-3-*O*-(2,3,4,6-tetra-*O*-benzyl-α-D-galactopyranosyl)-α-D-mannopyranoside (**9**, 70%). Mercury(II) cyanide in nitromethane–benzene is an effective catalyst for such condensation reactions affording 1,2-*trans* glycoside[8,12].

Hydrogenolysis of **8** gave methyl 3-*O*-α-D-galactopyranosyl-α-D-mannopyranoside (**10**), and removal of protecting groups from **9** gave methyl 3-*O*-α-D-galactopyranosyl-2-*O*-(β-D-glucopyranosyluronic acid)-α-D-mannopyranoside (**11**). Methyl 3-*O*-β-D-galactopyranosyl-α-D-mannopyranoside (**14**) was prepared by condensing **4** with 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide in the presence of mercury(II) cyanide and mercury(II) bromide in acetonitrile[16] followed by removal of protecting groups.



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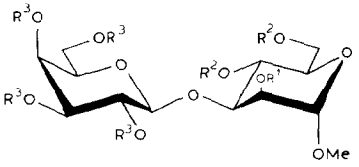

13 R^1 = Allyl, R^2 = Bzl, R^3 = Ac

14 $R^1 = R^2 = R^3 = H$

Methylation analysis of **10** gave 2,3,4,6-tetra-*O*-methylgalactose and 2,4,6-tri-*O*-methylmannose, and of **11** gave 2,3,4,6-tetra-*O*-methylgalactose and 4,6-di-*O*-methylmannose, which were identified by g.l.c. of the alditol acetates.

The antiserum used in the homologous precipitin reaction was raised[14] against killed whole cells of *Klebsiella* Type 10 in rabbits. The serum was brought to pH 6 with dilute hydrochloric acid and set up with increasing amounts of saline. After 4 days at +2°, the precipitates were removed by centrifugation and the clear supernatant solutions were examined for antibody excess by the Oüchterlony double-diffusion method[15]. The results showed that, at the equivalence point, 350 μg of the polysaccharide precipitated 595 μg of the antibody nitrogen from 1 mL of the antiserum. The results of the precipitin reactions are summarised in Table I. Methyl β-D-galactopyranoside, methyl α-D-mannopyranoside, D-glucuronic acid, and methyl 3-*O*-β-D-galactopyranosyl-α-D-mannopyranoside were inactive, **10** and **11** caused 70% and 75% inhibition respectively and were effective at low concentrations, and **12** caused 52.5% inhibition at a somewhat higher concentration. The amounts of the inhibitors needed to cause 50% inhibition and 28% inhibition are also given in Table I for the sake of comparison, although <50% inhibition is not significant. The amounts of methyl β-D-galactopyranoside, methyl α-D-mannopyranoside, and **14** necessary to achieve 28% inhibition* were >10 times that of **10**

*Inhibition to this extent may also be due to the presence of methyl β-D-galactopyranside and methyl α-D-mannopyranoside in the repeating unit of K-10.

TABLE I

INHIBITION OF ANTIGEN–ANTIBODY PRECIPITIN IN THE *Klebsiella* TYPE 10 IMMUNE SYSTEM

Inhibitor	*Inhibitor added*[a] *(μM)*	*Antibody nitrogen precipitated (μg)*	*Inhibition*[b] *(%)*	*Inhibitor needed*[c] *(μM)*	
				for 50% inhibition	*for 28% inhibition*
None		595			
Methyl α-D-galactopyranoside	10.0	286	52.0	9.0	3.0
Methyl β-D-galactopyranoside	11.0	428	28.0		12.0
Methyl α-D-mannopyranoside	14.0	417	30.0		13.0
D-Glucuronic acid	12.0	342	43.0		6.2
Methyl 3-*O*-α-D-galactopyranosyl-α-D-mannopyranoside (**10**)	2.5	179	70.0	2.1	1.5
Methyl 3-*O*-α-D-galactopyranosyl-2-*O*-(β-D-glucopyranosyluronic acid)-α-D-mannopyranoside (**11**)	1.8	149	75.0	1.3	1.0
2-*O*-(β-D-Glucopyranosyluronic acid)-D-mannopyranose (**12**)	3.5	283	52.0	3.3	2.1
Methyl 3-*O*-β-D-galactopyranosyl-α-D-mannopyranoside (**14**)	11.0	410	31.0		10.0

[a]Work was done on undiluted serum. [b]Average of duplicate runs. [c]Figures of 50% and 28% inhibition were obtained directly from the graph of % inhibition *vs.* inhibitor added.

and **11**. Moreover, methyl α-D-galactopyranoside was a much better inhibitor than the β anomer. Likewise, **10** was a much better inhibitor than **14**. These results were expected because methyl α-D-galactopyranoside, **10**, and **12** are components of **11**.

Thus, it is confirmed that methyl 3-*O*-α-D-galactopyranosyl-2-*O*-(β-D-glucopyranosyluronic acid)-α-D-mannopyranoside (**11**) is a part of the repeating unit of *Klebsiella* K-10 polysaccharide as in structure **1**.

EXPERIMENTAL

Materials and methods. — All reactions were monitored by t.l.c. on Silica Gel G (Merck). Column chromatography was performed on Silica Gel 60 (Merck). All solvents were distilled before use. Melting points were determined with a Fisher–Johns melting-point apparatus and are uncorrected.

P.c. was performed on Whatman No. 1 paper with *A*, 9:2:2 ethyl acetate–acetic acid–water; and *B*, 8:2:1 ethyl acetate–pyridine–water; with detection using alkaline silver nitrate. G.l.c. was performed at 180° for alditol acetates and 170° for partially methylated alditol acetates with a Hewlett–Packard Model 5730A instrument fitted with a Model 3380A electronic integrator and a glass column (1.83 m × 6 mm) packed with 3% of ECNSS-M on Gas Chrom Q (100–120 mesh).

Optical rotations were measured with a Perkin–Elmer Model 241MC spectropolarimeter. Absorbances were measured with a Hitachi Model 100-60 spectrophotometer. ^{1}H-N.m.r. spectra were recorded for solution in $CDCl_3$ (internal Me_4Si) with a Varian Model XL-200 spectrometer.

Methyl 2-O-allyl-4,6-di-O-benzyl-α-D-mannopyranoside (**4**). — To a solution of methyl 4,6-di-*O*-benzyl-α-D-mannopyranoside[4] (**3**, 1.5 g) in dichloromethane (75 mL) was added allyl bromide (0.36 mL), tetrabutylammonium bromide (0.32 g), and aqueous 5% sodium hydroxide (8 mL). The suspension was stirred vigorously for 2 days at room temperature, and the dichloromethane layer was then washed with water (4 × 50 mL), dried (Na_2SO_4), filtered, and concentrated to dryness. Column chromatography (benzene–ether, 5:4) of the syrupy residue gave **4** (1.25 g, 75%) together with the 3-*O*-allyl derivative (10%) and **3**. Compound **4** had $[\alpha]_D^{25}$ +48° (*c* 1, chloroform). ^{1}H-N.m.r. data ($CDCl_3$): δ 3.4 (s, 3 H, OMe), 5.1 (s, 1 H, H-1), 5.8–6.02 (m, 1 H, allylic proton), 7.3 (m, 10 H, 2 Ph).

Anal. Calc. for $C_{24}H_{30}O_6$: C, 69.54; H, 7.29. Found: C, 69.20; H, 7.08.

2,3,4,6-Tetra-O-benzyl-α-D-galactopyranosyl bromide (**6**). — Powdered potassium hydroxide (2 g) was stirred with a solution of ethyl 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-galactopyranoside[13] (2 g) in 1,4-dioxane (10 mL). Benzyl bromide (2 mL) was added dropwise during 2 h to the vigorously stirred solution at 80°. Heating and stirring were continued for another 4 h. The mixture was then cooled, diluted with water (10 mL), and extracted with chloroform (2 × 20 mL). The combined extracts were washed with water (4 × 15 mL), dried (Na_2SO_4), filtered, and concentrated to a syrup from which benzyl alcohol was removed by azeotropic distillation with water. Column chromatography of the residue gave pure ethyl

tetra-*O*-benzyl-1-thio-β-D-galactopyranoside (**5**; 2.1 g, 70%). Recrystallisation from ethanol gave material having m.p. 52–54°, $[\alpha]_D^{25}$ −12° (*c* 0.9, chloroform). ^{1}H-N.m.r. data ($CDCl_3$): δ 1.15–1.40 (t, 3 H, SCH_2CH_3), 2.5–2.9 (q, 2 H, SCH_2CH_3), 4.25 (d, 1 H, *J* 7.6 Hz, H-1), 7.15–7.35 (m, 20 H, 4 Ph).

Anal. Calc. for $C_{36}H_{40}O_5S$: C, 73.94; H, 6.89. Found: C, 73.42; H, 7.02.

A cooled solution of **5** (2 g) in dichloromethane (25 mL) was stirred with bromine (0.2 mL) for 15 min at 0°. The α-D-glycosyl bromide **6** was collected as a syrup after evaporation of the solvent *in vacuo* and used immediately.

Methyl 2-O-allyl-4,6-di-O-benzyl-3-O-(2,3,4,6-tetra-O-benzyl-α-D-galactopyranosyl)-α-D-mannopyranoside (**7**). — To a solution of **4** (1 g) in dichloromethane (15 mL) were added dry *N,N*-dimethylformamide (0.15 mL) and tetraethylammonium bromide (1 g). A solution of **6** (2 g) in dichloromethane (10 mL) was added with stirring under nitrogen. Triethylamine (2 mL) was added and stirring was continued for 2 days at room temperature. The mixture was partitioned between water and dichloromethane, and the organic layer was dried (Na_2SO_4), filtered, and concentrated to dryness. T.l.c. (benzene–ether, 9:1) showed one major and two minor components. The faster moving (major) component was isolated by column chromatography to give **7** (1.5 g, 65%), isolated as a syrup, $[\alpha]_D^{25}$ +61° (*c* 1.1, chloroform). ^{1}H-N.m.r. data ($CDCl_3$): δ 3.4 (s, 3 H, OMe), 5.1 (d, 1 H, *J* 1.5 Hz, H-1), 4.95 (d, 1 H, *J* 3.5 Hz, H-1′), 5.8–6.12 (m, 1 H, allylic H), 7.4 (m, 30 H, 6 Ph).

Anal. Calc. for $C_{58}H_{64}O_{11}$: C, 74.33; H, 6.88. Found: C, 74.02; H, 6.60.

Methyl 4,6-di-O-benzyl-3-O-(2,3,4,6-tetra-O-benzyl-α-D-galactopyranosyl)-α-D-mannopyranoside (**8**). — To a solution of **7** (1.3 g) in dry methyl sulfoxide (10 mL) was added potassium *tert*-butoxide (0.15 g), and the mixture was stirred under nitrogen for 2 h at 100°, then cooled, and diluted with water (20 mL). The mixture was extracted with chloroform (4 × 25 mL), and the combined extracts were washed with water (3 × 30 mL), dried (Na_2SO_4), filtered, and concentrated to dryness. To a solution of the residue in acetone–water (10:1, 11 mL) was added mercury(II) oxide (0.6 g) followed by a solution of mercury(II) chloride (0.6 g) in acetone–water (10:1, 11 mL) dropwise with stirring at room temperature. After 30 min, the mixture was filtered through Celite and concentrated to dryness. Ether (40 mL) was added and the solution was washed with saturated aqueous potassium iodide (20 mL) and water (2 × 20 mL), then concentrated to dryness. Column chromatography (benzene–ether, 8:1) of the syrupy residue gave **8** (0.76 g, 60%), isolated as a syrup, $[\alpha]_D^{25}$ +80° (*c* 1.8, chloroform). ^{1}H-N.m.r. data ($CDCl_3$): δ 3.4 (s, 3 H, OMe), 5.2 (d, 1 H, *J* 1.6 Hz, H-1), 4.97 (d, 1 H, *J* 3.6 Hz, H-1′), 7.38 (m, 30 H, 6 Ph).

Anal. Calc. for $C_{55}H_{60}O_{11}$: C, 73.6; H, 6.74. Found: C, 73.2; H, 6.95.

Methyl 4,6-di-O-benzyl-2-O-(methyl 2,3,4-tri-O-acetyl-β-D-glucopyranosyluronate)-3-O-(2,3,4,6-tetra-O-benzyl-α-D-galactopyranosyl)-α-D-mannopyranoside (**9**). — A solution of **8** (0.45 g) in 1:1 nitromethane–benzene (40 mL) was concentrated to 30 mL and cooled to 40°, and molecular sieve (4 Å, 2 g), mercury(II)

cyanide (0.2 g), and methyl (2,3,4-tri-*O*-acetyl-α-D-glucopyranosyl bromide)uronate[11] (0.4 g) were added, and stirring was continued for 24 h at 40° under nitrogen. More glycosyl bromide (0.2 g) and mercury(II) cyanide (0.1 g) were added and stirring was continued for 24 h. The solution was diluted with benzene, washed successively with water (0°), cooled saturated aqueous sodium hydrogencarbonate, and water, dried (Na_2SO_4), filtered, and concentrated to dryness under diminished pressure. Column chromatography (benzene–ether, 4:1) of the syrupy residue gave **9** (0.42 g, 70%). Crystallisation from ethanol–ethyl acetate (9:1) gave material having m.p. 141–142°, $[\alpha]_D^{25}$ +40° (*c* 2.5, chloroform). ^{1}H-N.m.r. data ($CDCl_3$): δ 1.8 (s, 3 H, OAc), 2.0 (2 s, 6 H, 2 OAc), 3.36 (s, 3 H, OMe), 3.8 (s, 3 H, COOMe), 4.85 (d, 1 H, *J* 7.5 Hz, H-1″), 4.95 (d, 1 H, *J* 3.6 Hz, H-1′), 5.3 (d, 1 H, *J* 1.5 Hz, H-1), 7.4 (m, 30 H, 6 Ph).

Anal. Calc. for $C_{68}H_{76}O_{20}$: C, 67.31; H, 6.31. Found: C, 67.22; H, 6.28.

*Methyl 3-*O*-α-*D*-galactopyranosyl-α-*D*-mannopyranoside* (**10**). — To a solution of **8** (225 mg) in dry methanol (5 mL) was added 10% Pd/C (0.5 g). The mixture was stirred under hydrogen for 36 h at room temperature, then filtered through Celite, and concentrated to dryness. P.c. (solvent *A*) of the residue gave **10** (66 mg, 74%), $[\alpha]_D^{25}$ +151° (*c* 0.5, water), $R_{Lactose}$ 1.2.

*Methyl 3-*O*-α-*D*-galactopyranosyl-2-*O*-(β-*D*-glucopyranosyluronic acid)-α-*D*-mannopyranoside* (**11**). — Compound **9** (200 mg) was *O*-debenzylated as described above. The product was stirred with methanolic 0.1M sodium methoxide (6 mL) for 3 h. Water (1 mL) was added, stirring was continued for a further 1 h, and the solution was decationised with Amberlite IR-120 (H^+) resin, filtered, and concentrated to dryness. P.c. (solvent *A*) of the product gave **11** (62 mg, 75%), $[\alpha]_D^{25}$ +128° (*c* 0.85, water), $R_{Lactose}$ 0.75.

*Methyl 3-*O*-β-*D*-galactopyranosyl-α-*D*-mannopyranoside* (**14**). — To a solution of **4** (2 g) in acetonitrile (25 mL) were added 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide (2.44 g), 3 Å molecular sieves (3 g), mercury(II) cyanide (1.52 g), and mercury(II) bromide (2.16 g) in succession. The mixture was stirred at room temperature for 24 h, then filtered through Celite, diluted with chloroform (150 mL), washed with water, aqueous 10% KI, saturated aqueous sodium hydrogencarbonate, and water, dried (Na_2SO_4), and concentrated. Column chromatography (benzene–ether, 5:1) of the syrupy residue gave methyl 2-*O*-allyl-4,6-di-*O*-benzyl-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-α-D-mannopyranoside (**13**; 2.02 g, 62%), $[\alpha]_D^{25}$ +32° (*c* 1.4, chloroform). ^{1}H-N.m.r. data ($CDCl_3$): δ 1.95–2.15 (4 s, 12 H, 4 Ac), 3.33 (s, 3 H, OMe), 3.7 (d, 2 H, O–CH_2–CH=CH_2), 4.8 (d, 1 H, *J* 7.6 Hz, H-1′), 5.15 (d, 1 H, *J* 1.5 Hz, H-1), 5.3 (d, 4 H, 2 PhCH_2), 5.6–6.0 (m, 1 H, CH_2CH=), 7.32–7.45 (m, 10 H, 2 Ph).

Anal. Calc. for $C_{38}H_{48}O_{15}$: C, 61.28; H, 6.50. Found: C, 61.15; H, 6.61.

To a solution of **13** (1.5 g) in 1-propanol–acetic acid–water[17] (2:1:1, 20 mL) was added 10% Pd/C (0.3 g). The mixture was stirred at 80° for 20 h, then cooled, filtered through Celite, and concentrated to dryness. The crude product was acetylated conventionally with pyridine and acetic anhydride. Column chromatography

(benzene–ether, 5:1) of the product gave methyl 2-*O*-acetyl-4,6-di-*O*-benzyl-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-α-D-mannopyranoside (0.72 g, 49%). ^{1}H-N.m.r. data ($CDCl_3$): δ 1.9–2.15 (5 s, 15 H, 5 Ac), 3.4 (s, 3 H, OMe), 4.85 (d, 1 H, *J* 8 Hz, H-1′), 5.00 (d, 1 H, *J* 1.5 Hz, H-1), 5.25 (d, 4 H, 2 PhCH_2), 7.35–7.45 (m, 10 H, 2 Ph).

The above compound (250 mg) was *O*-debenzylated and *O*-deacetylated as described above. P.c. (solvent *A*) of the residue gave **14** (92 mg, 71%), $[\alpha]_D^{25}$ +47° (*c* 0.6, water), $R_{Lactose}$ 1.25.

Acid hydrolysis and methylation analysis of **10, 11,** *and* **14**. — Acid hydrolysis and methylation analysis were carried out as described[1].

Characterisation of methyl 2-O-allyl-4,6-di-O-benzyl-α-D-mannopyranoside (**4**). — Compound **4**, after methylation[7], was *O*-deallylated[10], then *O*-debenzylated, and converted into the alditol acetate[1]. G.l.c. showed it to be a derivative of 3-*O*-methylmannose.

Preparation of rabbit antiserum. — Antiserum to *Klebsiella* K-10 was raised in rabbits as described[14].

Quantitative precipitin reaction. — The polysaccharide was added in increasing amounts (10–100 μg) to 0.1-mL portions of homologous K-10 antiserum, and each volume was made up to 0.5 mL with normal saline. The mixtures (in duplicate) were kept at 37° for 1 h and then, together with blanks containing serum only, kept at 0–2° for 96 h. Each was centrifuged for 1 h at 3000 r.p.m. at 0–2° and the supernatant solution was tested for excess of antibody in Oüchterlony plates. Each precipitate was washed thrice with chilled aqueous 0.9% sodium chloride and then dissolved in 3 mL of 0.25M acetic acid, and the absorbance was determined at 280 nm. The amount of antibody nitrogen precipitated was calculated from a standard curve calibrated by using rabbit IgG.

Inhibition studies. — To 0.1 mL of antiserum, in duplicate, were added increasing amounts of inhibitors, and the mixtures were stored at 0–2° for 1 h. An amount of antigen solution needed to bring the system to equivalence was then added to each mixture. The volume was made up to 0.5 mL with normal saline, and the mixture was kept at 37° for 1 h. Two sets of controls, one containing the same amount of antigen and antibody as in the other tubes and the other containing only the serum, were also included. The tubes were kept at 0–2° for 96 h and the amounts of precipitated nitrogen were calculated as described above. The same procedure was repeated for each inhibitor used.

ACKNOWLEDGMENTS

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