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Structure of the K10 capsular antigen from *Escherichia coli* O11 : K10 : H10, a polysaccharide containing 4,6-dideoxy-4-malonylamino-D-glucose

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ABSTRACT

The K10 antigen from *Escherichia coli* O11 : K10 : H10 consists of equimolar amounts of rhamnose and 4,6-dideoxy-4-malonylaminoglucose [Qui4NMal; 4-(2-carboxyacetamido)-4,6-dideoxyglucose]. Methylation analysis and 1 and 2D NMR spectroscopy showed that the K10 capsular polysaccharide has the structure

$$\rightarrow 3)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-Qui}p\text{4NMal-}(1\rightarrow$$
$$\overset{2|}{\text{OAc}}$$

INTRODUCTION

Escherichia coli, notably those causing extraintestinal infections, are frequently encapsulated. The capsular antigens (K antigens) are acidic polysaccharides[1–3] which, on the basis of genetic, biochemical, and structural parameters, have been classified into two groups[3]. Group I polysaccharides are expressed by the bacterial cells at all growth temperatures[4]. They are determined from a *his*-linked chromosomal gene cluster and have repeating units of tetra- to hexa-saccharides. Group II polysaccharides are expressed by the bacterial cells only at growth temperatures above 20–25°C[4]. They are determined from the *ser*A-linked chromosomal *kps* genes[1,3,5,6] and have di- to tri-saccharide repeating units[3]. The molecular organisation of the *kps* gene cluster is the same in all *E. coli* that express group II capsular polysaccharides[7,8]. A significant characteristic of *E. coli* with group II capsules is a greatly elevated activity of CMP-KDO synthetase[9,10]. The K10 polysaccharide of *E. coli* O11 : K10 : H10 is also determined from a *ser*-A linked gene cluster[11]. The organisation of the K10 genes is different from that of the group II *kps* genes (I.

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Roberts, personal communication). Furthermore, *E. coli* K10 does express the capsule at all growth temperatures and it does not exert an elevated activity of CMP-KDO synthetase. Thus, the K10 capsular polysaccharide seems to be a member of yet another polysaccharide group, to which the K3, K11, and K54 polysaccharides also belong[9]. To characterise the chemical nature of the K10 polysaccharide, we have elucidated its primary structure. The results are presented herein.

RESULTS AND DISCUSSION

Isolation and characterisation of the polysaccharide.—The K10 polysaccharide was isolated from liquid cultures of *E. coli* 21454 (O11 : K10 : H10) by a sequence[12,13] that involved precipitation with cetyltrimethylammonium bromide (CTAB), extraction with aqueous calcium chloride, precipitation with ethanol, and removal of contaminating protein by extraction with cold phenol (pH 6.5). The K10 polysaccharide was obtained in a yield of 30–40 mg per litre of culture. It contained L-rhamnose, as identified with L-rhamnose isomerase[14], and an acidic component **1** which, on acid hydrolysis, was converted into a basic component **2**. The polysaccharide also contained ca. 0.8 mol equiv of *O*-acetyl, as determined from signal intensities in an inverse gated ^{13}C NMR spectrum. The ^{13}C NMR spectrum (Fig. 1) of the *O*-deacetylated polysaccharide contained, *inter alia*, signals for anomeric carbon atoms (δ 101.6 and 104.5), 6-deoxy methyl groups (δ 17.9, double intensity), carbonyl groups of amide and/or carboxylate (δ 173.9 and 172.3), and a nitrogen-

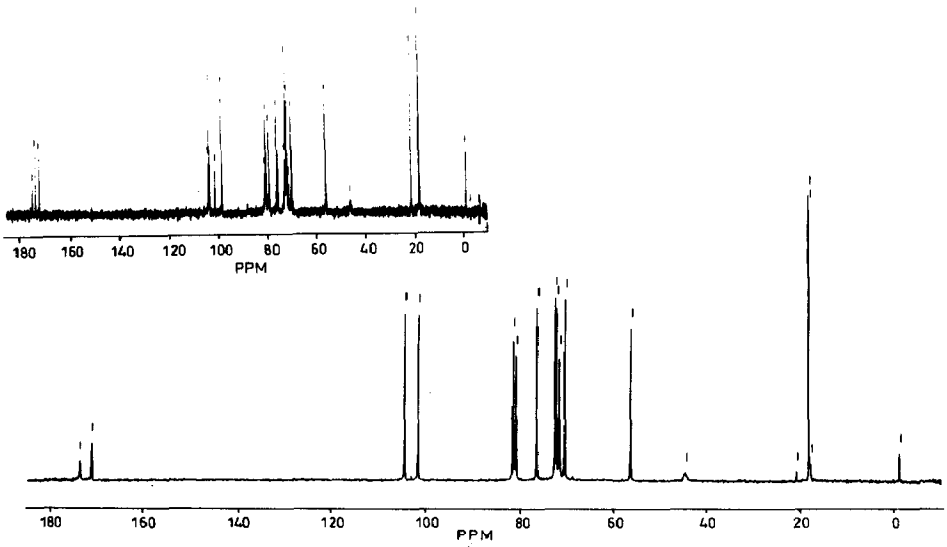
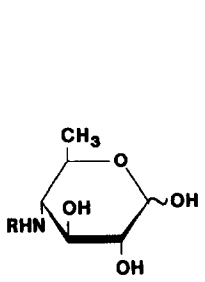
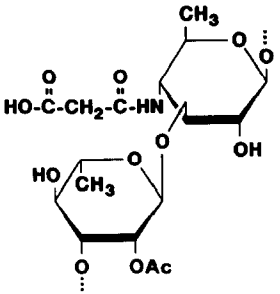


Fig. 1. 75-MHz ^{13}C NMR spectra of the native (inset) and *O*-deacetylated K10 polysaccharides, recorded in D_2O (60°C) with acetone (δ 31.45) as internal standard.



1 R= CO-CH2-COOH

2 R= H



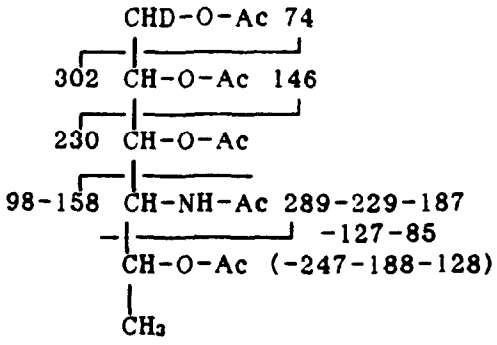
→3)-α-L-Rhap-(1→3)-β-D-Quip-4NMal-(1→
2|
OAc

3

bearing carbon atom (δ 56.4). A broad signal at δ 46.1 indicated a $-CO-CH_2-CO-$ group. The ^1H NMR spectrum contained signals due to the anomeric proton of a residue with a *manno* configuration (δ 5.14) and a residue with a *gluco* or *galacto* configuration (δ 4.60; $J_{H\text{-}1,H\text{-}2}$ 7.8 Hz). A signal arising from the methyl groups of 6-deoxy sugars (δ 1.20) was also apparent.

Characterisation of the acidic component **1**.—The acidic component of the K10 polysaccharide was liberated by mild acid hydrolysis and purified by ion-exchange chromatography. In high-voltage paper electrophoresis, **1** migrated anodically with M_{GlcA} 0.87. After acid hydrolysis (0.5 M sulfuric acid, 100°C, 16 h) and subsequent neutralisation of the hydrolysate, **2** was obtained, which migrated in high-voltage electrophoresis as a cation with a mobility of M_{GlcN} 1.09. It was reduced with sodium borodeuteride, acetylated with acetic anhydride–pyridine, and analysed by GLC–MS. From the mass spectrum shown in Fig. 2, the product was identified as 4-acetamido-1,2,3,5-tetra-*O*-acetyl-4,6-dideoxy-1-deuteriohexitol. For the determination of configuration, **2** was converted into its phenylthiocarbamoyl derivative by reaction with phenyl isothiocyanate. The product was compared in reversed-phase HPLC with the phenylthiocarbamoyl derivatives of 4-amino-4,6-dideoxyhexoses of known configuration. The results (Table I) indicated that **2** was 4-amino-4,6-dideoxyglucose. For further characterisation of **1**, the K10 polysaccharide was carboxyl-reduced with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide–sodium borohydride[15]. The reduced polysaccharide was hydrolysed (1 M trifluoroacetic acid, 100°C, 90 min) and, after neutralisation, the mixture of sugar components was reduced with sodium borohydride and peracetylated. In subsequent GLC–MS analysis, a substance was obtained whose mass spectrum and fragmentation pattern are shown in Fig. 3. The results indicated that **1** was 4,6-dideoxy-4-malonylaminoglucose [4-(2-carboxyacetamido)-4,6-dideoxyglucose].

For an independent characterisation of malonate, the *O*-deacetylated K10 polysaccharide was hydrolysed (0.5 M HCl, 80°C, 16 h) and the neutralised hydrolysate was analysed by thin-layer chromatography. Detection with chromosul-



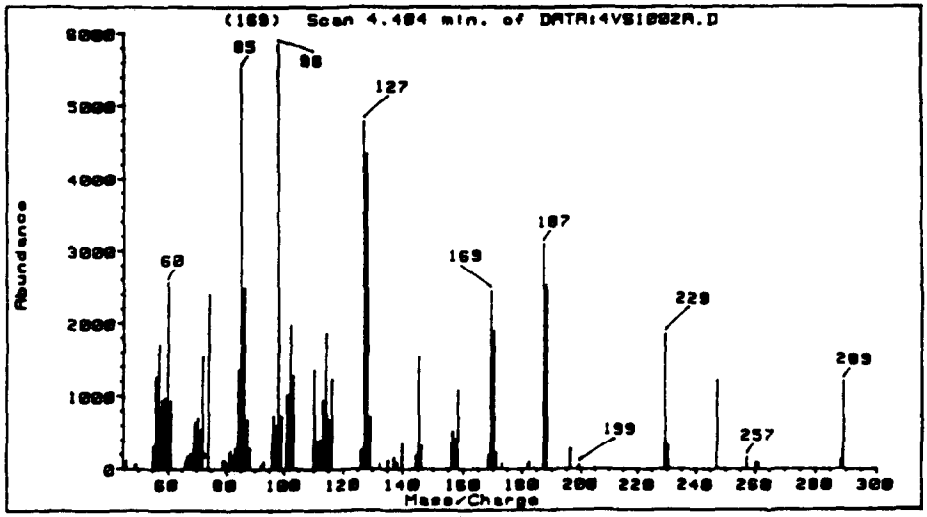


Fig. 2. Mass spectrum and fragmentation pattern of per-*O*-acetylated 4-acetamido-4,6-dideoxyglucitol-*1-d*.

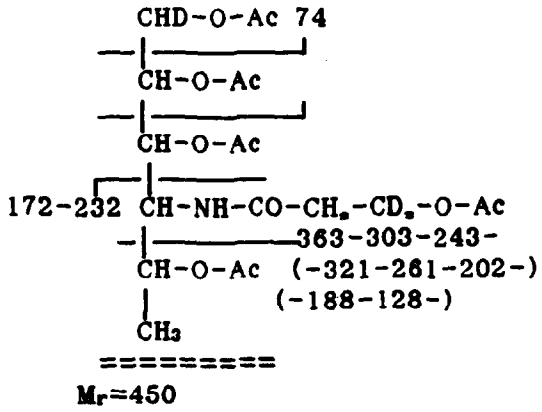
furic acid[16] revealed a spot with the same mobility (R_f 0.27) as that of authentic malonate[17].

Methylation analysis.—The K10 polysaccharide was methylated with KH–Me_2SO and MeI in Me_2SO[18]. The purified (Sep-Pak C_{18} cartridges) product was

TABLE I

Reversed-phase HPLC of *N*-phenylthiocarbamoyl derivatives of 4-amino-4,6-dideoxy sugars on Bondapak C_{18}

4-Amino-4,6-dideoxyhexose	Polysaccharide source	Retention time (min)	References
Unknown	K10	5.83	
D-*gluco*-	O7	5.89	20, 25
D-*galacto*-	O10	6.35	20
D-*manno*-	O157	6.46	26



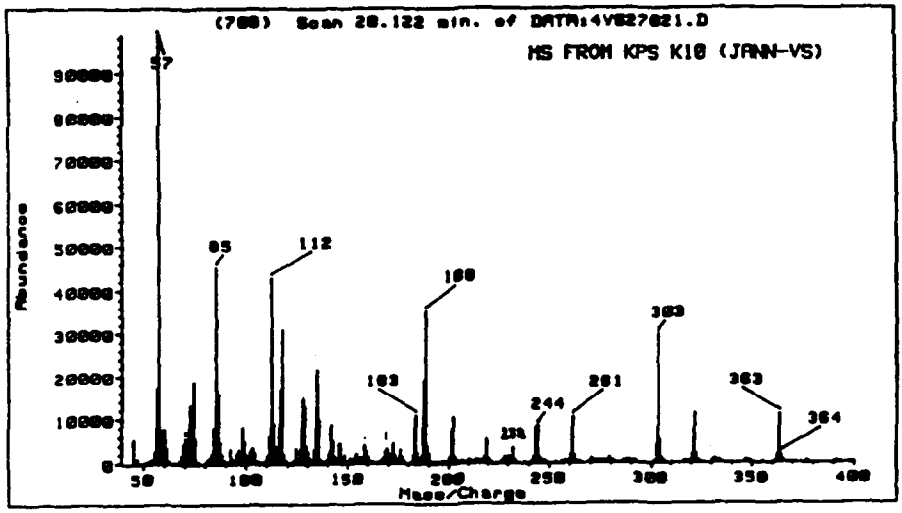
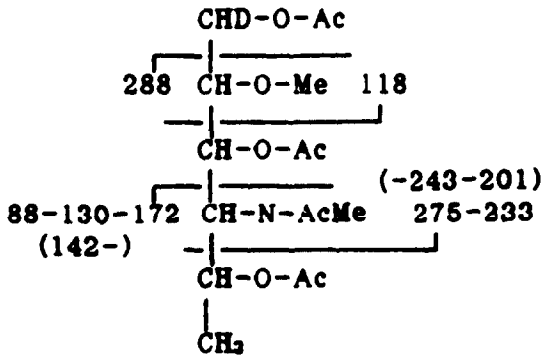


Fig. 3. Mass spectrum and fragmentation pattern of per-*O*-acetylated 4,6-dideoxy-4-(3-hydroxypropionamido-*3,3-d*$_2$-glucitol-*1-d*.

hydrolysed[19] and the products were reduced with sodium borodeuteride, then *O*-acetylated. GLC (ECNSS-M) gave two products which were shown by EIMS to be 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methylrhamnitol-*1-d* and 1,3,5-tri-*O*-acetyl-4,6-dideoxy-2-*O*-methyl-4-(*N*-methylacetamido)glucitol-*1-d*. The mass spectrum and fragmentation pattern of the latter compound are shown in Fig. 4. These results showed that both constituents of the K10 polysaccharides were 3-linked.

NMR spectroscopy.—For an assignment of the ring protons in both sugar constituents, a 2D-NMR analysis was performed. Fig. 5 shows the results of a H,H COSY-RELAY experiment. The data were used to assign the signals of the ^{13}C NMR spectrum in a 2D heteronuclear COSY experiment (Fig. 6). Since no significant cross-peaks for H-4 of the amino sugar moiety could be found in the COSY NMR spectra, the characteristic signal of C-4 of this sugar in the ^{13}C NMR spectrum was used for the assignment of the H-4 signal. The assignments of the



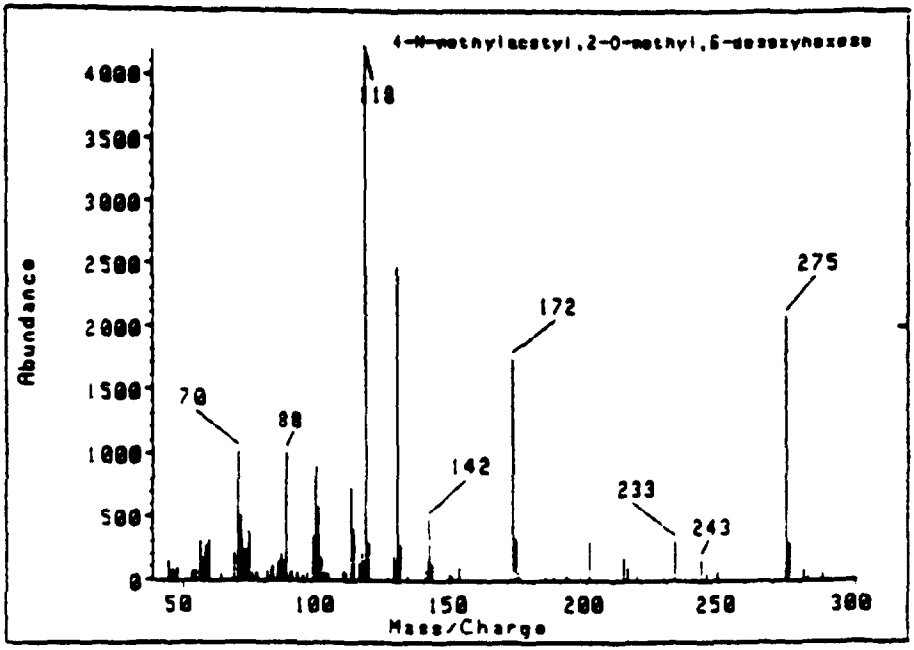


Fig. 4. Mass spectrum and fragmentation pattern of 1,3,5-tri-*O*-acetyl-4,6-dideoxy-2-*O*-methyl-4-(*N*-methylacetamido)glucitol-*1-d*.

^{13}C NMR signals are shown in Table II. The ^{13}C NMR data for the sugar part of the acidic component **1** in the *E. coli* K10 polysaccharide compare well with those reported for the sugar part of the 4-acetamido-4,6-dideoxyglucose residue in the *E. coli* O7 polysaccharide[20]; the signal assignments obtained for the sugar part of isolated **1** were identical with those reported for 4,6-dideoxy-4-(glycylamino)glucose from *Shigella dysenteriae* type 7 polysaccharide[21].

The D configuration of 4,6-dideoxy-4-malonylaminoglucose was determined[22] from the glycosylation effects exerted on C-2 of L-rhamnose (−0.3 ppm) and on C-1 of the *N*-acylamino sugar (+7.7 ppm).

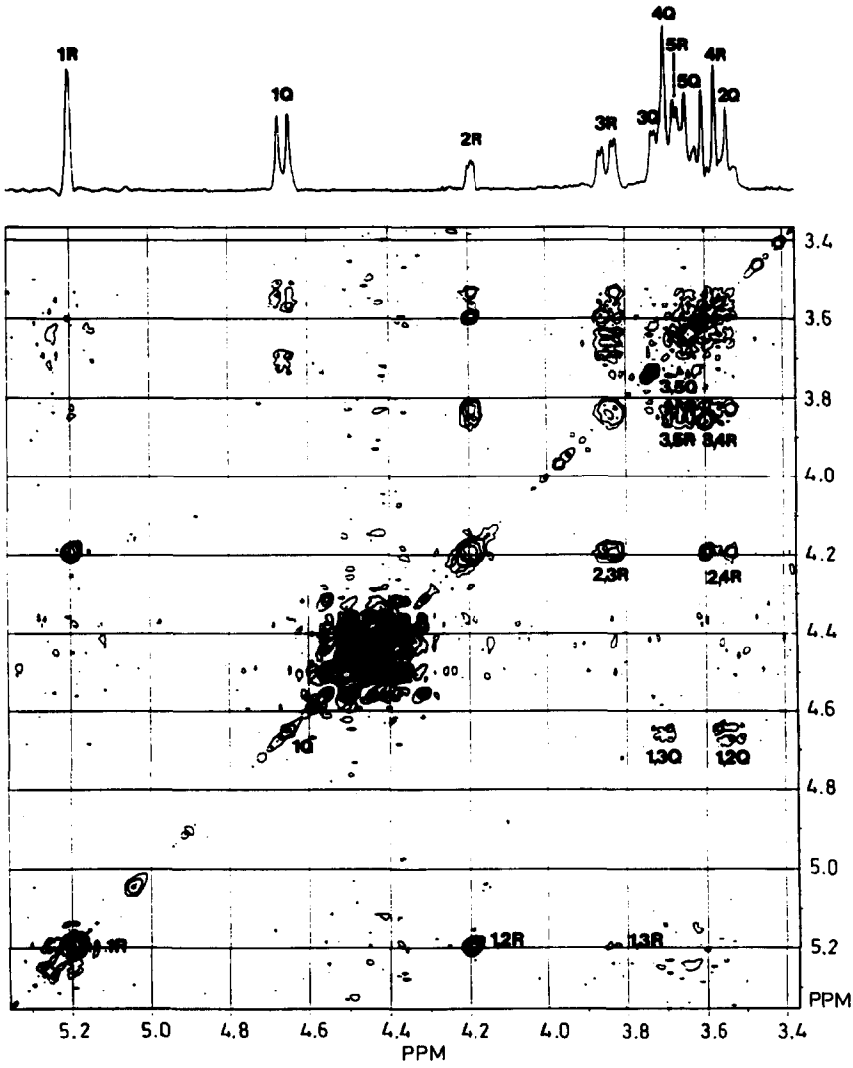


Fig. 5. 2D H,H COSY-RELAY spectrum of the *O*-deacetylated K10 polysaccharide.

The native polysaccharide showed 3 signals in the anomeric region. One of these (δ 98.6) disappeared on *O*-deacetylation, whereas another one (δ 101.6, assigned to α-rhamnose) increased in intensity. This indicated *O*-acetylation at C-2 of the rhamnose residue.

The results of NMR spectroscopy are in agreement with those of chemical analysis. The K10 polysaccharide, which can be formulated as **3**, belongs to the group of capsular polysaccharides with small repeating units. It is noteworthy that the negative charge is due to *N*-acylation with malonic acid and thus caused by a non-carbohydrate constituent. Whereas substitution with an amino acid has been

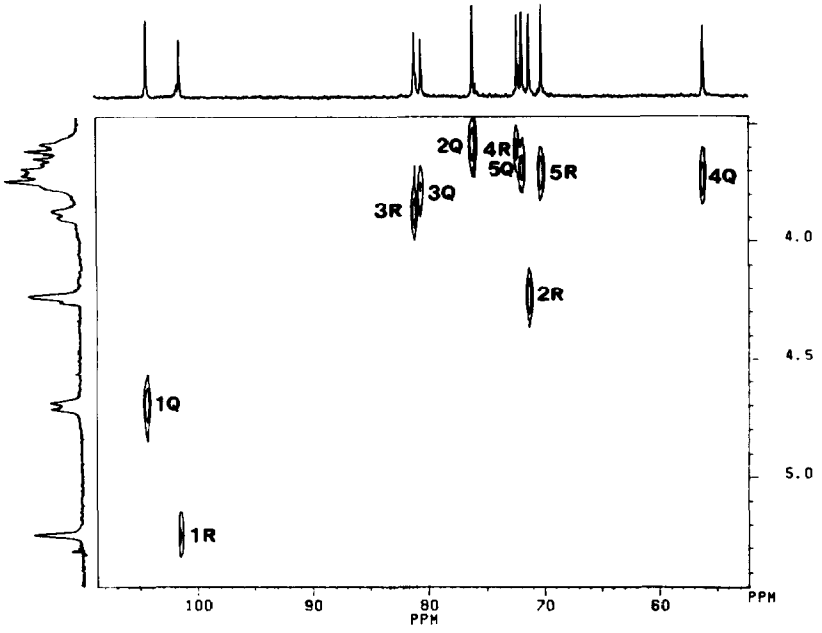


Fig. 6. 2D ^{13}C–^{1}H COSY spectrum of the *O*-deacetylated K10 polysaccharide.

found with *E. coli* capsular polysaccharides[3,23], substitution with a divalent acid has not been reported previously for this group of polysaccharides.

EXPERIMENTAL

Bacteria and cultivation.—E. coli 21454 (O11 : K10 : H10) was grown to the late logarithmic phase in a fermenter in 10-L batches, which contained per litre: $K_2HPO_4 \cdot 3H_2O$ (9.6 g), KH_2PO_4 (2 g), sodium citrate $\cdot 5H_2O$ (0.5 g), $MgSO_4 \cdot 7H_2O$ (0.1 g), casamino acids (20 g), ammonium sulfate (1 g), D-glucose (2 g), and the dialysable part of yeast (100 mL from 500 g in 5 L of deionised water).

TABLE II

Signal assignments of the ^{13}C NMR spectrum of the K10 polysaccharide

Unit	C-1	C-2	C-3	C-4	C-5	C-6	M_{C-1} [a]	M_{C-2} [a]	M_{C-3} [a]
→ 3)-Rha	101.6	71.4	81.2	72.4	70.3	17.9			
→ 3)-Qui4NMal [b]	104.5	76.2	80.7	56.4	72.0	17.9	173.9 [c]	46.1	172.5 [c]

[a] Carbon atoms of the malonyl substituent. [b] 4,6-Dideoxy-4-malonylaminoglucose. [c] Values are interchangeable.

Isolation and purification of the capsular polysaccharide.—The acidic capsular K10 polysaccharide and the bacterial cells were precipitated from the liquid culture by addition of 1 vol of aq 2% CTAB. All following operations were carried out at 4°C. The polysaccharide was extracted from the precipitate with M $CaCl_2$, and purified by three cycles of precipitation from aqueous solution with EtOH (to 80% final concentration), followed by repeated extractions with cold aq 80% phenol (buffered to pH 6.7 with sodium acetate)[12,13]. The combined aqueous phases were centrifuged for 4 h (100000*g*) and the supernatant solution was lyophilised. The residue was further purified on a column of Sephadex G-50 with water as eluant.

Carboxyl reduction.—The carboxyl groups were reduced[15,23] in the intact polysaccharide by first reacting in aqueous solution with a tenfold excess of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide at pH 4.57 and subsequent reduction of the active ester formed with $NaBH_4$. After decomposition of excess of $NaBH_4$, the solution was dialysed against deionised water and freeze-dried.

Methylation.—The polysaccharide and the carboxyl-reduced polysaccharide were methylated[18] with Me_2SO–KH–MeI in Me_2SO and the product was solvolysed[19] with H_2SO_4–glacial acetic acid (1:19, 16 h, 80°C). For GLC–MS analysis, the hydrolysis products were reduced with $NaBH_4$ and peracetylated with Ac_2O–pyridine as described[18,19].

Isolation of **1**.—For the isolation of the acidic component **1**, the polysaccharide (100 mg) was hydrolysed (1 M CF_3CO_2H, 1 h, 100°C). The neutralised hydrolysate was chromatographed on AG-1-X8 with 1 M sodium acetate as eluant. After desalting (Dowex 50, H^+), **1** was obtained from the eluate by lyophilisation (51 mg).

Analytical methods.—Rhamnose was determined with the cysteine–H_2SO_4 reagent[24] and with L-rhamnose isomerase[14]. The latter reaction was also used for the determination of the absolute configuration of L-rhamnose. *N*-Phenylthiocarbamoyl derivatives of 4-amino-4,6-dideoxyhexoses were characterised by reversed-phase HPLC (Waters PICO-TAG amino acid analysis system) on Bondapak C_{18}, according to the producers' manual). Malonate was detected[16,17] by thin-layer chromatography on Silica Gel 60 with 1-propanol–H_2O–concd aq NH_3 (6:3:1) as solvent.

4-Acetamido-4,6-dideoxyglucose and its peracetate were quantitated by inverse gated ^{13}C NMR spectroscopy, using characteristic signals (NH–COCH_3, C–CH_3, O–CH_3) as reporter groups. GLC–MS was carried out with a Hewlett–Packard 5988A instrument, using a DB 5 capillary column (0.2 mm × 30 m) with He as carrier gas starting at 30°C with a temperature program of 50°C/min and then from 180–250°C at 5°C/min. NMR spectra were recorded for solutions in D_2O with a Bruker WM 300 spectrometer at 300 MHz (^1H NMR) or at 75 MHz (^{13}C NMR) with acetone as internal standard (δ 2.23 for ^1H spectra; δ 31.45 for ^{13}C spectra). Standard Bruker software was used for 2D COSY, COSYRTC, and COSYRTC2.

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