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Structural studies of the capsular polysaccharide from *Klebsiella* type 38: a reinvestigation

Per-Erik Jansson [a], Bengt Lindberg [a], M. Cristina Manca [a,1], Wolfgang Nimmich [b], Göran Widmalm [a,*]

[a] *Department of Organic Chemistry, Arrhenius Laboratory, Stockholm University, S-10691 Stockholm, Sweden*

[b] *Institut für Medizinische Mikrobiologie, Universität Rostock, D-2500 Rostock, Germany*

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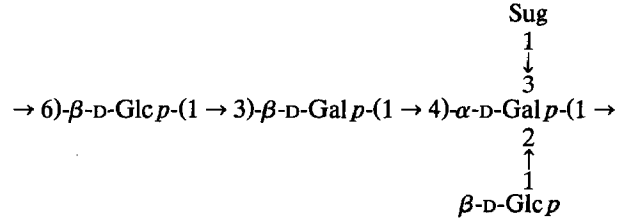
Abstract

The structure of the capsular polysaccharide from *Klebsiella* type K38 has been reinvestigated. It is composed of pentasaccharide repeating units of the structure given below. In this structure, Sug stands for a 4-deoxy-*threo*-hex-4-enopyranosyluronic acid group, most probably having the β-L configuration. ^{1}H NMR studies further indicate that this group assumes the $^1H_2$ conformation.



Key words: Klebsiella; Bacterial; Polysaccharide; 4-Deoxy-*threo*-hex-4-enopyranosyluronic acid

* Corresponding author.

[1] On leave from: Istituto per la Chimica di Molecole di Interesse Biologico del C.N.R., Arco Felice, 80072 Napoli, Italy.

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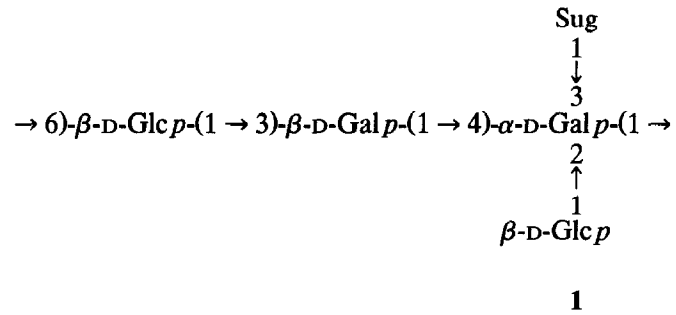
1. Introduction

Some 20 years ago, we proposed that the capsular polysaccharide elaborated by *Klebsiella* type 38 (K38) was composed of pentasaccharide repeating units having the structure **1** [1].



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In this structure, Sug stands for a 3-deoxy-L-*glycero*-pentulofuranosylonic acid group. The acidic sugar was removed by hydrolysis on treatment with acid under mild conditions. The structure of the modified polysaccharide was determined by conventional methods, namely, methylation analysis, characterisation of products obtained on partial acid hydrolysis of the polysaccharide and the methylated polysaccharide, and Smith degradation.

The characterisation of the acidic sugar was based upon circumstantial evidence only. It gave a positive reaction with the periodate–barbituric acid reagent, and 2-furoic acid was formed on treatment with acid, as expected for a 3-deoxypentulosonic acid. The negative $[\alpha]_D$ value of its 2,4-dinitrophenylhydrazone indicated that it had the L-*glycero* configuration.

When this investigation was performed, we had no access to FT-NMR spectroscopy. Recently recorded ^1H and ^{13}C NMR spectra of K38 revealed that the proposed structure was incorrect. The ^1H NMR spectrum contained, *inter alia*, signals at δ 6.07, 5.42, 5.16, 4.73, 4.63, and 4.61, and only the last four of these are accounted for as anomeric protons by the proposed structure. The structure of K38 has therefore been reinvestigated.

2. Results and discussion

Sugar analysis of K38 gave D-glucose and D-galactose in equal amounts, and these sugars accounted for ~80% of the polysaccharide material. Methylation analysis of K38 gave 2,3,4,6-tetra-*O*-methyl-D-glucose, 2,4,6-tri-*O*-methyl-D-galactose, 2,3,4-tri-*O*-methyl-D-glucose, and 6-*O*-methyl-D-galactose. In the methylation analysis of the polysaccharide obtained after hydrolysis of K38 with acid under mild conditions, 6-*O*-methyl-D-galactose was replaced by 3,6-di-*O*-methyl-D-galactose. These results are the same as in the previous investigation [1], and support the proposed structure (**1**), except for the nature of the acidic component. ^{13}C

NMR spectra of the latter polymer showed signals for 24 carbons, in agreement with a tetrasaccharide repeating unit of four hexopyranosides. The signals for anomeric carbons were observed at δ 104.9, 104.7, 104.4, and 99.1. The proposed structure of the tetrasaccharide repeating unit was further corroborated by a NOESY experiment on the polysaccharide obtained after hydrolysis of K38 with acid under mild conditions. NOE contacts were observed, *inter alia*, between H-1 (δ 4.63) of the terminal glucosyl group and H-2 (δ 4.04) of the 2,4-substituted α-linked galactosyl residue, and between H-1 (δ 4.66) of the 3-substituted β-linked galactosyl residue and H-4 (δ 4.26) of the 2,4-substituted α-linked galactosyl residue, thereby supporting the proposed structure.

The ^{1}H NMR spectrum of K38 (Fig. 1) contained five signals in the region δ 4.5–5.5, assigned to anomeric protons (see below), and a signal at δ 6.07 (1 H). The ^{13}C NMR spectrum (Fig. 2) contained six signals in the region for anomeric carbons, at δ 107.6, 104.8, 104.4, 103.9, 102.3, and 99.2, a signal in the carbonyl region at δ 169.2, and signal at δ 146.0, an unusual chemical shift for a polysaccharide. These values indicate that K38 contains an unusual component. ^{1}H,^{1}H-Correlated spectroscopy shows that the anomeric signals at δ 4.61 ($J_{1,2}$ 7.3 Hz), 4.63 ($J_{1,2}$ 8.0 Hz), and 4.73 ($J_{1,2}$ 7.6 Hz) are derived from hexopyranosides that have the β configuration. The signal at δ 6.07 was a double doublet with $J_{H,H}$ 4.6 and 1.4 Hz. A ^{1}H,^{1}H-COSY spectrum (Fig. 3) revealed that this signal and that at δ 5.42 ($J_{H,H}$ 2.8 Hz) belong to the same spin system by correlations at δ 4.03 and 4.18, assigned to H-3 and H-2, *vide infra*, respectively. The signal at δ 6.07 showed cross-peaks in the ^{1}H,^{1}H-COSY spectrum to δ 4.03 (strong) and 4.18 (medium), whereas that at δ 5.42 showed cross-peaks to δ 4.18 (strong) and 4.03 (very weak). The signal at δ 5.42 is thus assigned to H-1 and that at δ 6.07 to H-4. The signal at δ 5.16 ($\nu_{1/2}$ 7 Hz) was assigned to the remaining hexopyranoside, which should be α-linked. The coupling pathways were corroborated by relayed COSY experiments. Thus, a component of K38 contains a four-proton spin system. The chemical shifts of C-1

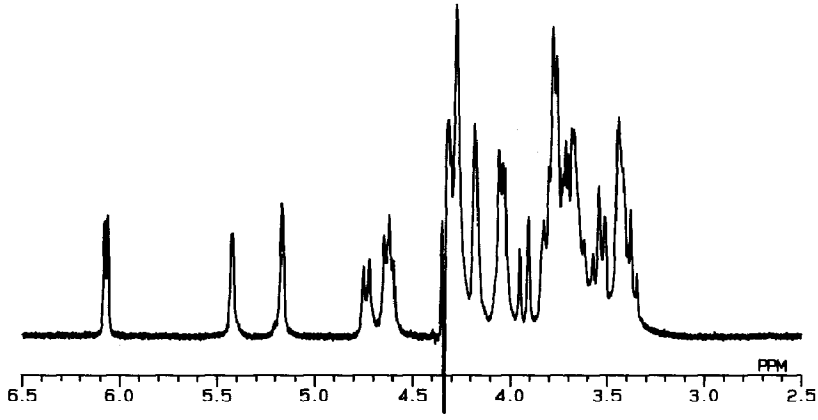


Fig. 1. The ^{1}H NMR spectrum of the *Klebsiella* K38 capsular polysaccharide.

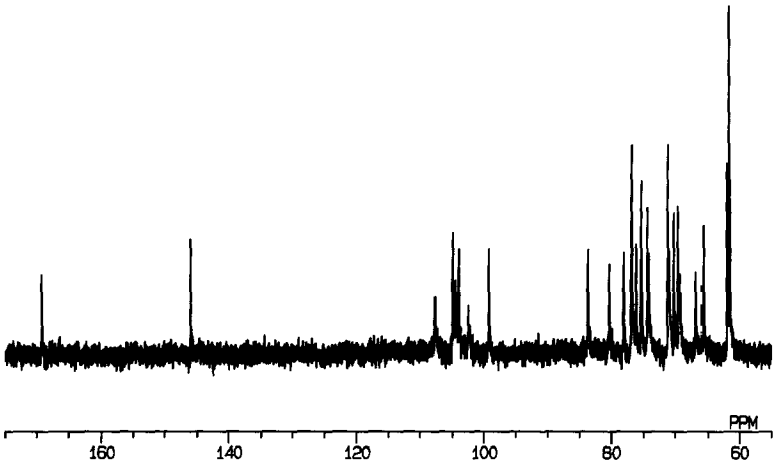


Fig. 2. The ^{13}C NMR spectrum of the *Klebsiella* K38 capsular polysaccharide.

and C-4, δ 102.3 and 107.6, respectively, were determined by $^{1}H,^{13}C$-correlated spectroscopy. From an HMBC-experiment, by which long-range proton to carbon correlations can be observed, C-5 (δ 146.0) and C-6 (δ 169.2) of the component could be assigned. The correlations were, *inter alia*, H-4 to C-5 and C-6 as well as H-1 to C-5. These results indicate that the unknown component in K38 is a 4-deoxyhex-4-enopyranosyluronic acid. Groups of this type are formed on treatment of polysaccharides containing 4-substituted hexopyranosyluronic acid residues, e.g., heparin [2] and the *Klebsiella* K14 [3] and K64 [4] capsular polysaccharides, with lyases. Such groups are also formed on treatment of the *Klebsiella* K22 capsular polysaccharide, which contains terminal 4-*O*-[(*S*)-1-carboxyethyl]-β-D-glucopyranosyluronic acid groups, with a bacteriophage-borne enzyme [5]. There were also indications that native K22 contains some 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid groups.

Heparin that had been degraded with heparin lyase and contained 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid end groups was carboxyl-reduced, hydrolysed with acid in the presence of 4-methylmorpholine–borane [6], in order to reduce the labile 4-deoxy-L-*threo*-hexos-5-ulose (**3**) as soon as it is formed, and acetylated, yielding a product which should contain acetylated 4-deoxy-D-*xylo*- and 4-deoxy-L-*arabino*-hexitol (or more correctly 3-deoxy-L-*xylo*- and 3-deoxy-L-*lyxo*-hexitol) (**4**). These were not separated in GLC, but gave the expected mass spectrum with the strong secondary ions of m/z 69 and 81. (The origin of some primary fragments is indicated in the formula.) On similar treatment of carboxyl-reduced K38, a component with the same retention time and mass spectrum was obtained. The component from K38 which had been carboxyl-reduced using sodium borodeuteride showed the expected shifts of pertinent ions, as indicated in parentheses in formula **4**. These experiments therefore confirm that the acidic sugar in K38 is a 4-deoxy-hex-4-enopyranosyluronic acid. The 4-deoxyhexitol acetate mixture pre-

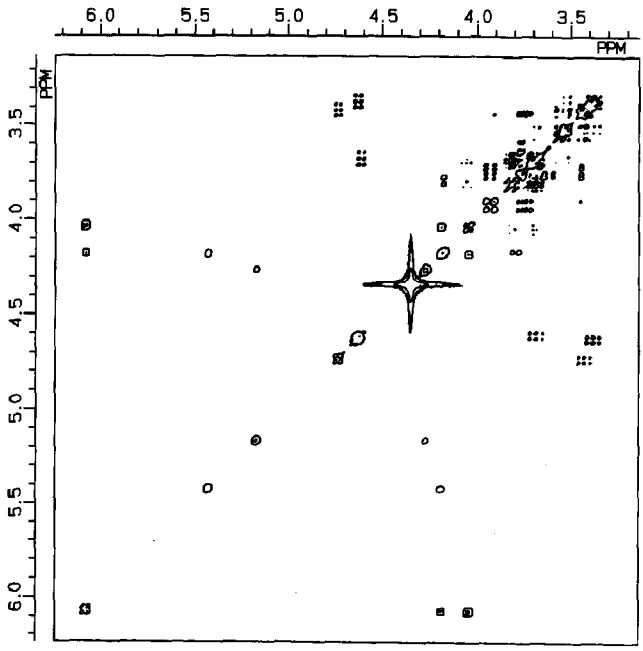
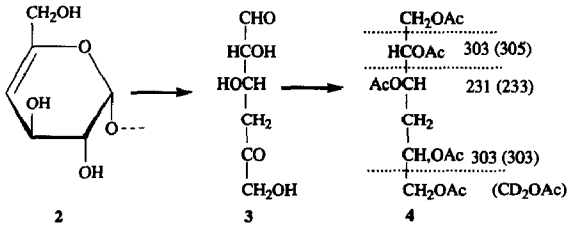


Fig. 3. The $^{1}H-^{1}H$ COSY spectrum of the *Klebsiella* K38 capsular polysaccharide.

pared from K38 had higher retention time in GLC than 4-deoxy-L-*ribo*-hexitol acetate, prepared from 3-deoxy-D-*ribo*-hexose. This result demonstrates that the former, and the 4-deoxyhex-4-enopyranosyluronic acid in K38, have the *threo* configuration at C-2 and C-3 (Scheme 1).

The biosynthesis of the 4-deoxyhex-4-enopyranosyluronic acid in K38 almost certainly involves β-elimination from a uronic acid derivative having a *threo* relationship between HO-2 and HO-3, most probably at the sugar nucleotide level. The four uronic acids with this L-*threo* relationship, namely those with D-*gluco*, D-*galacto*, L-*ido*, and L-*altro* configurations, all occur as components in bacterial polysaccharides [7], whereas the corresponding enantiomers have not been found



Scheme 1.

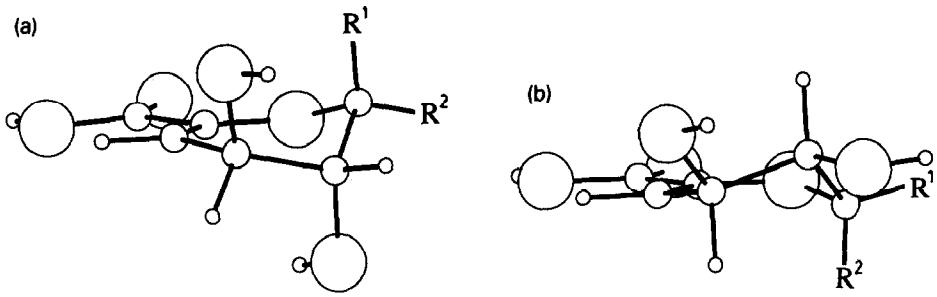


Fig. 4. The two different half-chair forms, $^1H_2$ (a) and $^2H_1$ (b) of a 4-deoxy-L-*threo*-hex-4-enopyranosyluronic acid group. The α configuration has $R^1 = O\cdots$ and $R^2 = H$, whereas the β configuration has $R^1 = H$ and $R^2 = O\cdots$.

in Nature. It therefore seems reasonable to assume that the 4-deoxyhex-4-enopyranosyluronic acid in K38 has the L-*threo* configuration.

A 4-deoxy-L-*threo*-hex-4-enopyranosyluronic acid group may assume two different half-chair forms, $^1H_2$ (Fig. 4a) and $^2H_1$ (Fig. 4b) [8]. In the $^2H_1$ conformation, there is a *trans*-diaxial relationship between H-2 and H-3, which should lead to a large $J_{2,3}$. This is, however, not observed ($J_{2,3} \sim 3$ Hz) for the group in K38, and thus the $^1H_2$ conformation should predominate. The α-L-*threo* form in the $^2H_1$ conformation should also show a large $J_{1,2}$, due to the *trans*-diaxial relationship between H-1 and H-2. The observed coupling constants are, however, $J_{1,2}$ 2.8, $J_{3,4}$ 4.6, and $J_{2,4}$ 1.4 Hz. The $J_{1,2}$ and $J_{3,4}$ values are similar to those observed by Ragazzi et al. [8] for the α anomer in the $^1H_2$ conformation, as present in a disaccharide from chondroitin sulfate. For the α-L-*threo* form in the $^1H_2$ conformation, both H-1 to H-3 and H-2 to H-4 assume a *W* arrangement [9], for which $^4J_{H,H}$ values > 1 Hz are expected, and have also been reported [8]. For the 4-deoxy-L-*threo*-hex-4-enopyranosyluronic acid group in K38, only $J_{2,4}$ is significant, 1.4 Hz, thus indicating a β configuration. Further support of the β configuration of Sug comes from similarity of its C-4 (δ 107.6) and C-5 (δ 146.0) chemical shifts to those of a 4-deoxy-β-L-*threo*-hex-4-enopyranosyluronic acid group from lyase-degraded capsular polysaccharide from *Klebsiella* K64 [4] where the chemical shifts are δ 109.3 and 145.6 for C-4 and C-5, respectively. In contrast, the chemical shifts of a 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid group from the lyase-degraded capsular polysaccharide of *Klebsiella* K14 [3] are δ 112.0 and 141.9 for C-4 and C-5, respectively.

From the combined evidence, it is proposed that K38 is composed of pentasaccharide repeating units with the structure **1**. In this structure, Sug stands for a 4-deoxy-*threo*-hex-4-enopyranosyluronic acid group, most probably having the β-L configuration.

The formation of 2-furoic acid on acid hydrolysis of K38 was confirmed. A substance with the same mobility in TLC was isolated, and had the same ^{1}H NMR spectrum as the authentic substance. A mechanistic interpretation of this reaction,

which should involve loss of carbon monoxide or formic acid at some stage, must await further studies.

3. Experimental

General methods.—Concentrations were performed under diminished pressure at < 40°C or under a stream of air. For GLC, a Hewlett–Packard 5890 instrument fitted with a flame-ionisation detector was used. GLC–MS(EI) was performed on a Hewlett–Packard 5970 MSD instrument. Alditol acetates and partially methylated alditol acetates were analysed on an HP-5 capillary column (25 m × 0.20 mm × 0.33 μm), using the temperature program 180°C (1 min) → 250°C at 3°C/min.

NMR spectroscopy.—NMR spectra of solutions in D_2O were recorded at 70°C with a Jeol GSX-270 instrument. Chemical shifts are reported in ppm, using sodium 3-trimethylsilylpropanoate-d_4 (δ_H 0.00) and acetone (δ_C 31.00) as internal references. 2D NMR spectroscopy was performed using Jeol standard pulse-sequences.

Sugar and methylation analyses.—Methylation was carried out essentially as described earlier [10]. Carboxyl reduction was performed according to Taylor et al. [11]. Hydrolysis of carboxyl-reduced K38 was performed with aq 1% AcOH at 70°C for 2.5 h, in the presence of 4-methylmorpholine–borane [6].

Isolation of the neutral polysaccharide.—A solution of K38 (13 mg) in aq 1% AcOH (5 mL) was kept at 70°C for 2.5 h, neutralised, and dialysed against water, and the polymer was purified by FPLC chromatography on a Superdex 30 prep grade column (Pharmacia, Uppsala, Sweden).

Acknowledgments

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