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Structural studies of the capsular polysaccharide from *Klebsiella* type 7

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Abstract

The structure of the capsular polysaccharide elaborated by *Klebsiella* type 7 has been investigated. NMR spectroscopy together with sugar and methylation analysis have been the main methods used. A uronic acid degradation was also employed. The polysaccharide consists of hexasaccharide repeating units having the following structure.

→3)-β-D-Glc*p*A-(1→2)-α-D-Man*p*-(1→2)-α-D-Man*p*-(1→3)-β-D-Glc*p*-(1→3)-4,6-(*S*)-Pyr-β-D-Glc*p*-(1→
3
↑
1
α-D-Gal*p*

Keywords: *Klebsiella*; Capsular polysaccharide

1. Introduction

In the genus *Klebsiella*, some 80 capsular (K) antigens have been described on a serological basis of these type-specific polysaccharides [1], and most of the structures

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Table 1
Methylation analysis of K7 and some modified products [a]

Sugar		Detector response %		
	T	A	B	C
2,3,4,6-Gal [b]	1.00	17	16	19
3,4,6-Man	1.26	24	14	22
2,4,6-Glc	1.27	22	21	21
2,4,6-Man	1.32			18 [d]
4,6-Man	1.64	15	18	
2,4-Glc	1.89		19 [c]	
2-Glc	2.30	22	12	20

[a] Key: *T*, retention time relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-galactitol; A, methylated CPS; B, carboxyl-reduced and methylated CPS; C, uronic acid-degraded CPS.
[b] 2,3,4,6-Gal = 2,3,4,6-tetra-*O*-methyl-D-galactose, etc.
[c] Deuterium-labelled at C-6.
[d] Trideuteriomethyl at O-2.

have been elucidated [2]. For the type 7 antigen only a partial structure has been published [3]. Here we report on the structure of the repeating unit of the capsular polysaccharide from K7.

2. Results and discussion

Hydrolysis of the capsular polysaccharide from *Klebsiella* serotype 7 (K7) with trifluoroacetic acid yielded mannose, glucose, and galactose in the proportions 30:47:23. The presence of glucuronic acid was deduced from GLC analysis of a sample that had been treated with methanol and methanolic hydrogen chloride followed by acetylation. The absolute configurations were determined by GLC of the acetylated (+)-2-butyl glycosides and were D for all the constituents. The presence of D-glucuronic acid was thus further corroborated.

Methylation analysis of K7 (Table 1, column A) revealed terminal galactose, 2-substituted mannose, 3-substituted glucose, 2,3-disubstituted mannose, and 3,4,6-trisubstituted glucose. Reduction of methylated K7 with lithium triethylborodeuteride yielded, after hydrolysis, an ether corresponding to a 3-substituted glucuronic acid in addition to the residues mentioned above (Table 1, column B). These results indicate that K7 is composed of hexasaccharide repeating units. Together with NMR data (vide infra) the methylation analysis also shows that all sugar components are pyranoid.

Methylated K7 was subjected to a uronic acid degradation [4], that is, treatment with base followed by addition of trideuteriomethyl iodide and standard methylation analysis workup (Table 1, column C). During this treatment the glycosidic linkage of the D-glucuronic acid should be cleaved and the hydroxyl group in the released sugar residue trideuteriomethylated. The appearance of a new methyl ether, 2,4,6-tri-*O*-methyl-D-mannose with a trideuteriomethyl group at O-2, and the disappearance of 4,6-di-*O*-methyl-D-mannose demonstrate structural element **1**.

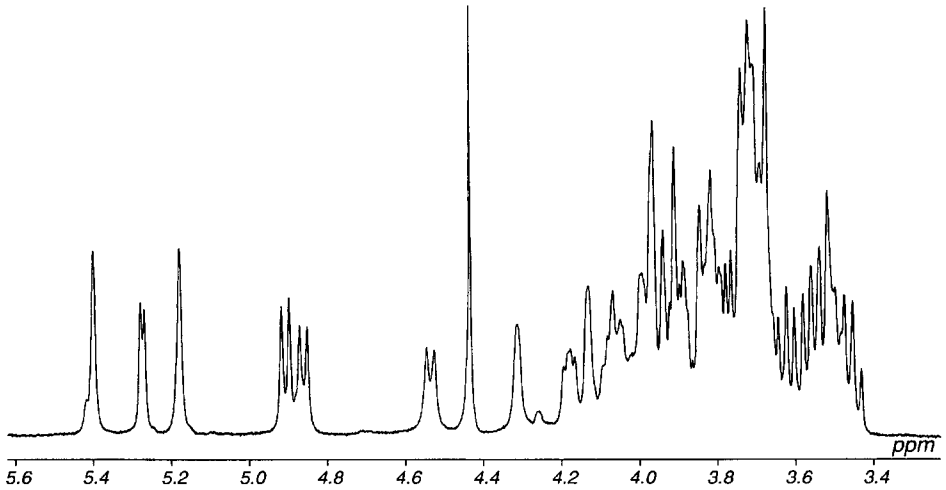
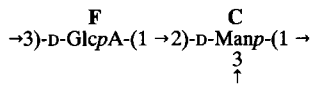


Fig. 1. The 3.3–5.6 ppm region in the ^{1}H NMR spectrum of K7.



1

Standard methylation analysis workup after base treatment gave results identical to those of the ordinary methylation analysis.

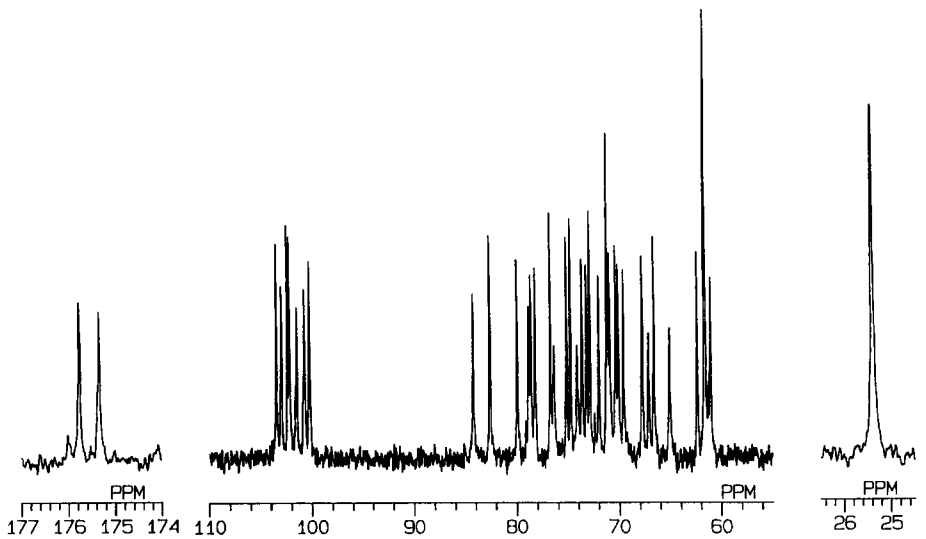


Fig. 2. The ^{13}C NMR spectrum of K7.

Table 2
Chemical shifts (ppm) of the signals in the ^1H and ^{13}C NMR spectra [a] of K7

Sugar residue	H/C 1	2	3	4	5	6a	6b
→ 2)-α-D-Man*p*-(1 →	5.40	4.13	3.98	3.74	4.00 [c]	n.a. [b]	n.a.
A	100.2 (4.8) [d][174]	78.6	71.1	67.7	73.6	61.7	
α-D-Gal*p*-(1 →	5.28	3.79	3.91	3.97	4.18	~ 3.73	~ 3.73
B	101.5 (3.7)[174]	69.6	70.1	70.3	72.0	62.4	
→ 2)-α-D-Man*p*-(1 →	5.18	4.31	4.05	3.91	3.73	n.a.	n.a.
C 3 ↑	100.7 (5.3) [d][173]	78.8	76.3	67.1	74.1	61.1	
→ 3)-β-D-Glc*p*-(1 →	4.91	3.45	3.69	3.54	3.59 [c]	3.93	n.a.
D	102.2 (7.7)[164]	72.8	82.6	71.1 [e]	76.7	61.7	
→ 3)-4,6-(*S*)-Pyr-β-D-Glc*p*-(1 →	4.86	3.62	3.96	3.58	3.50	3.69 [f]	4.07 [g]
E	103.5 (7.7)[166]	75.1	79.9	74.7	66.6	65.1	
→ 3)-β-D-Glc*p*A-(1 →	4.54	3.54	3.72	3.66 [c]	3.68 [c]		
F	103.0 (8.1)[161]	73.2 [e]	84.2	70.9	78.2	175.8 [c]	

[a] $J_{H\text{-}1,H\text{-}2}$ values in Hz in parenthesis and $J_{C\text{-}1,H\text{-}1}$ values in Hz in square brackets.
[b] n.a. = not assigned.
[c] Tentative assignments.
[d] $\nu_{1/2}$.
[e] Interchangeable.
[f] *pro-S*.
[g] *pro-R*.

The ^1H and ^{13}C NMR spectra of K7 (Figs 1 and 2) indicate the presence of hexasaccharide repeating units and one pyruvate group. The spectra were assigned using 2D NMR spectroscopy and the assignments are given in Table 2. The sugar components are labelled **A**–**F** with respect to decreasing chemical shift of their respective anomeric proton, **A** and **C** having *manno* configuration because of their small (not resolved) $J_{H\text{-}1,H\text{-}2}$ values, and the rest, **B**, **D**, **E**, **F**, having *gluco / galacto* configuration because of their larger values of $J_{H\text{-}1,H\text{-}2}$.

A ^{13}C–^1H coupled ^1H-detected HMQC experiment [5,6] revealed one-bond ^{13}C–^1H coupling constants of 174 and 173 Hz for the anomeric proton signals of units **A** and **C**, respectively, indicating that the mannose residues are α-linked. The intermediate $J_{H\text{-}1,H\text{-}2}$ value of 3.7 Hz for the anomeric proton of **B** suggests that it is α-linked as well. **D**–**F** all show large values of $J_{H\text{-}1,H\text{-}2}$ (7.7–8.1 Hz) for the signals from anomeric protons and are thus β-linked.

A and **C** are the 2- and 2,3-substituted α-mannose residues, respectively, as judged by $\delta_{C\text{-}2}$, $\delta_{C\text{-}3}$, $\delta_{H\text{-}2}$, $\delta_{H\text{-}3}$, **C** showing downfield shifts for both positions whereas for **A** this only occurs for position two. Chemical shifts of signals from C-2–C-5 of unit **B** match those of α-galactopyranose [7], indicating terminal galactose. The resonances at δ_H 1.48/δ_C 25.4, δ_C 102.4, and δ_C 175.3 (tentatively assigned) are indicative of a

pyruvate group having the *S* configuration linked through O-4 and O-6 of a pyranosidic hexose [8,9]. Since the carbon and proton resonances 3, 4, and 6 of unit **E** are shifted downfield and 2-*O*-methyl-D-glucose is demonstrated in the methylation analysis, it is concluded that residue **E** is a 3-substituted glucose with a pyruvate group linked to O-4 and O-6. The two remaining residues are both 3-substituted pyranosides having the *gluco* configuration, one being glucose and the other glucuronic acid. From ^{1}H,^{1}H-HOHAHA experiments [10], using spin lock times of up to 150 ms, a cross-peak was detected in the experiment with the longest spin lock time, at δ_H 3.93, originating from the anomeric proton at δ_H 4.91 (unit **D**), whereas only cross-peaks at $\delta_H < 3.75$ can be observed from the anomeric signal at δ_H 4.54 (unit **F**). In a ^{13}C,^{1}H-COSY spectrum the signal at δ_H 3.93 does not display any correlation with a signal from a ring carbon, but to a signal for a primary carbon at δ_C 61.7, which indicates that the proton signal originates from an H-6. Thus **D** is the glucose residue and **F** is the glucuronic acid residue. The glucuronic acid residue has been shown to be linked to position 2 of the 2,3-disubstituted mannose residue, but it was not clear at this stage which residue is linked to the 3-position of the mannose residue **C**; it could be either an α-D or a β-D sugar. Some conclusions about the assignments made above can, however, be drawn from K7 NMR data. Two compounds with similar stereochemical arrangements around the glycosidic linkages, methyl 3-*O*-α-L-fucopyranosyl-4-*O*-β-L-fucopyranosyl-α-D-galactopyranoside and methyl 3-*O*-β-L-fucopyranosyl-4-*O*-β-L-fucopyranosyl-α-D-galactopyranoside, have been studied by NMR spectroscopy [11]. These compounds are mirror images of the two possible trisaccharide branch-points in K7 and show the same type of atom–atom interactions. It has been shown for both these trisaccharides that the chemical shift displacement of H-1 of the 4-O-substituent, corresponding to the 2-O-substituent in K7, is upfield. The upfield displacement for H-1 of residue **F** is 0.11 ppm, in agreement with the models. For H-1 of residue **D** a downfield shift of 0.27 ppm is observed. This further demonstrates that **D** is the glucose residue and **F** is the glucuronic acid residue. The downfield chemical shifts of their C-3s are in agreement with both units being 3-substituted.

Further assignments were corroborated from intra-residue correlations via $^{2}J_{C,H}$ and $^{3}J_{C,H}$ couplings from an HMBC experiment [12]. These were, inter alia, H-4 (δ 3.74) in **A** to its C-6 (δ 61.7), H-5 (δ 4.18) in **B** to its C-6 (δ 62.4), H-2 (δ 4.31) in **C** to its C-4 (δ 67.1), H-4 (δ 3.91) in **C** to its C-6 (δ 61.1), H-2 (δ 3.45) in **D** to its C-3 (δ 82.6), H-4 (δ 3.54) in **D** to its C-6 (δ 61.7), C-5 (δ 76.7) in **D** to its H-4 (δ 3.54), C-5 (δ 66.6) in **E** to its H-4 (δ 3.58), H-6b (δ 4.07) in **E** to its C-4 (δ 74.7) and to the acetal carbon (δ 102.4) of the pyruvate group. In the spectrum of methyl 4,6-(*S*)-Pyr-β-D-Glc*p* one of the signals for H-6 protons is found at δ_H 4.05 [9] and has a $J_{H\text{-}5,H\text{-}6}$ value of 4.8 Hz. The corresponding proton must thus be H-$6_{pro\text{-}R}$. The signal for H-$6_{pro\text{-}S}$ is observed at δ_H 3.73. In K7, the signal for one of the H-6 protons in **E** is observed at δ_H 4.07, that is, at almost the same chemical shift as the *pro-R* proton in the model substance. It is therefore assigned to *pro-R*. Furthermore, this proton shows $^{3}J_{C,H}$ coupling, in the HMBC spectrum, to C-2 in the pyruvate group and to C-4 in **E**, which is expected as these atoms are *trans* to each other [13].

The remaining sequence of sugar residues was determined using NOESY (see Table 3) and ^{1}H-detected HMBC (see Table 4) experiments to establish inter-residue through

Table 3
Observed NOE from anomeric protons of K7

Residue	Anomeric proton δ_H	NOE to proton δ_H	Residue, atom
→ 2)-α-D-Man*p*-(1 →	5.40	5.18	**C**, H-1
A		4.13	**A**, H-2
		3.69	**D**, H-3
α-D-Gal*p*-(1 →	5.28	4.05	**C**, H-3
B		3.79	**B**, H-2
→ 2)-α-D-Man*p*-(1 →	5.18	4.54	**F**, H-1
C 3		4.31	**C**, H-2
↑		4.13	**A**, H-2
→ 3)-β-D-Glc*p*-(1 →	4.91	3.96	**E**, H-3
D		3.69	**D**, H-3
→ 3)-4,6-(*S*)-Pyr-β-D-Glc*p*-(1 →	4.86	3.96	**E**, H-3
E			
→ 3)-β-D-Glc*p*A-(1 →	4.54	4.31	**C**, H-2
F		3.68	**F**, H-5

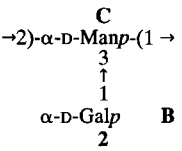
space proximity and through-bond connectivities, respectively. It should be noted that for α-linked pyranosides $^3J_{H,C}$ correlations in an HMBC experiment are normally observed from H-1 to C-3 and C-5 as well as over the glycosidic linkage, while for β-linked pyranosides the $^3J_{H,C}$ correlations are usually only detected across the glycosidic linkage [13].

Table 4
Observed $^3J_{H,C}$ connectivities in HMBC experiments for the anomeric atoms of K7

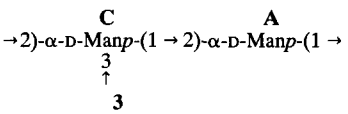
Residue	Anomeric atom		$J_{H,C}$ connectivities to		Residue, atom
	δ_H	δ_C	δ_C	δ_H	
→ 2)-α-D-Man*p*-(1 →	5.40		82.6		**D**, C-3
A	5.40		73.6		**A**, C-5
	5.40		71.1		**A**, C-3
		100.2		3.69	**D**, H-3
α-D-Gal*p*-(1 →	5.28		76.3		**C**, C-3
B	5.28		72.0		**B**, C-5
	5.28		70.1		**B**, C-3
→ 2)-α-D-Man*p*-(1 →	5.18		~ 78.7		**A**, C-2 and/or
C 3					**C**, C-2
↑	5.18		76.3		**C**, C-3
	5.18		74.1		**C**, C-5
		100.7		4.13	**A**, H-2
→ 3)-β-D-Glc*p*-(1 →	4.91		79.9		**E**, C-3
D					
→ 3)-4,6-(*S*)-Pyr-β-D-Glc*p*-(1 →	4.86		84.2		**F**, C-3
E					
→ 3)-β-D-Glc*p*A-(1 →	4.54		~ 78.7		**C**, C-2
F					

Structural element **1** was corroborated by an inter-residue NOE from H-1 in **F** to H-2 in **C** as well as by a long-range correlation in the HMBC spectrum for which only one assignment is in accord with the above data, namely, from H-1 in **F** to C-2 in **C**.

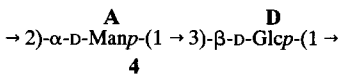
The branch-point α-D-Man residue is substituted at its 3-position by the terminal α-D-Gal group as seen by an NOE from H-1 in **B** to H-3 in **C**. The HMBC spectrum showed a long-range correlation from H-1 in **B** to C-3 in **C**, further demonstrating disaccharide element **2**.



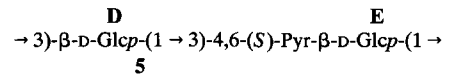
The branch-point residue is linked to the 2-substituted α-D-Man residue because inter-residue NOEs from H-1 in **C** to H-1 and H-2 in **A** were observed, as well as a long-range connectivity from C-1 in **C** to H-2 in **A**, defining structural element **3**. In agreement with the latter structural element a cross-peak was observed in the HMBC spectrum, which originates from a correlation between H-1 in **C** and C-2 in **A**.



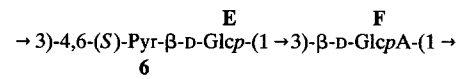
The 2-substituted mannose residue substitutes position 3 of the glucose residue as an inter-residue NOE from H-1 in **A** to H-3 in **D** was detected. HMBC correlations were also observed from C-1 and H-1 in **A** to H-3 and C-3 in **D**, respectively, establishing disaccharide element **4**.



The 3-substituted glucose residue in turn substitutes position 3 of the pyruvate-bearing glucose residue as an inter-residue NOE is observed from H-1 in **D** to H-3 in **E** along with a long-range HMBC correlation detected from H-1 in **D** to C-3 in **E**, defining structural element **5**. A β-D-glucose residue linked to position three of a D-glucose residue is expected to have a rather small downfield chemical shift displacement of the H-1 signal [14]. The large downfield shift of the H-1 signal of residue **D** may be caused by the proximity of H-1 in **D** to an oxygen atom in the pyruvate-bearing residue **E**.

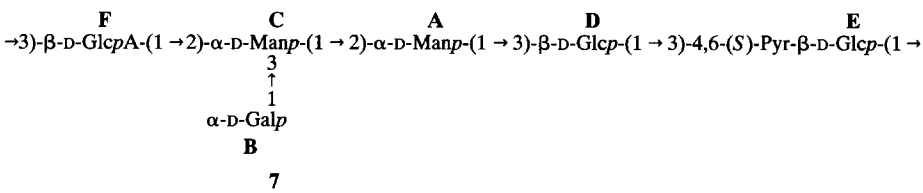


Finally the long-range HMBC connectivity between H-1 in **E** and C-3 in **F** gives the last disaccharide element, namely, **6**.



6

The sequence of the six residues is therefore **B-C-A-D-E-F-**, starting from the terminal galactose. The hexasaccharide repeating unit of Klebsiella K7 capsular polysaccharide is thus defined by **7**.



7

K7 cross-reacts with *Klebsiella* K10 antigen [1]. The common partial structure β-D-Glc*p*A-(1 → 2)[α-D-Gal*p*-(1 → 3)]-α-D-Man*p*-(1 → [15], which is the branching trisaccharide, is probably responsible for the cross-reaction. Another cross-reaction with an unknown origin [1] has been described with the capsular polysaccharide of *Escherichia coli* K55 [16].

3. Experimental

General methods.—Concentrations were performed under diminished pressure at < 40°C or under a stream of air or N_2. For GLC, a Hewlett–Packard 5890 instrument fitted with a flame-ionisation detector was used. GLC-MS was performed on a Hewlett–Packard 5970 MSD instrument. In both cases an HP-5 capillary column was used.

Alditol acetates and acetylated (+)-2-butyl glycosides were analysed using the temperature program 180°C (1 min) → 250°C at 3°C/min and partially methylated alditol acetates were analysed using the temperature program 170°C (1 min) → 180°C at 1°C/min, 180°C (1 min) → 210°C at 4°C/min.

NMR spectroscopy.—NMR spectra of solutions in D_2O were recorded at 60°C using either a JEOL GSX-270 or alpha 400 spectrometer and standard JEOL pulse sequences. Chemical shifts are reported in ppm referred to internal standards sodium 3-(trimethylsilyl)-[2,2,3,3-$^2H_4$]propanoate (TSP, δ_H 0.00) and acetone (δ_C 31.00). Chemical shifts were obtained from 1D spectra when possible, or from proton–proton correlated 2D COSY, relayed COSY, or ^1H,^1H-HOHAHA spectra. Data processing was performed using standard JEOL software or Felix 2.05 (Biosym Technologies, San Diego, CA, USA).

Relayed COSY spectra were obtained using a delay time of 30 or 60 ms. $^{1}H,^{1}H$-HOHAHA experiments were performed in the phase-sensitive mode and obtained using mixing times of 60, 90, 120 and 150 ms. The $^{1}J_{C\text{-}1,H\text{-}1}$ values were determined by an HMQC inverse detected experiment, and the $^{1}H-^{13}C$ long-range couplings were investigated with an HMBC inverse detected experiment using delay times of 45 and 90 ms. NOESY experiments were performed using mixing times of 150 and 350 ms.

Isolation of the polysaccharide.—Isolation of the *Klebsiella* K7 CPS (strain Aerogenes 4140 from the International Escherichia and Klebsiella Centre, Statens Seruminstitut, Copenhagen, Denmark) was performed as described previously [17].

Sugar and methylation analysis.—Methylation was carried out essentially according to methods described earlier [18,19]. Carefully dried methylated polysaccharide (1.5 mg) in dry THF (0.2 mL) was treated with M lithium triethylborodeuteride in THF (0.2 mL) at 0°C for 1 h to obtain the methylated carboxyl-reduced polysaccharide. The native and methylated polysaccharide were each treated with 0.5 M trifluoroacetic acid at 100°C overnight. The sugars in the hydrolysates were then converted into the alditol acetates or partially methylated alditol acetates. The absolute configurations of the sugars in a hydrolysate, obtained by treatment with 0.5 M trifluoroacetic acid at 90°C overnight, were determined essentially as devised by Leontein et al. [20], but using (+)-2-butanol.

Uronic acid degradation.—Carefully dried methylated polysaccharide (1.5 mg) in Me_2SO (1.5 mL) was treated with a trace of *p*-toluenesulfonic acid and 2,2-dimethoxypropane (0.1 mL). Sodium methylsulfinylmethanide (2 M, 1 mL) was then added at room temperature and the solution left overnight. After cooling, trideuteriomethyl iodide (0.5 mL) was added and the mixture agitated on an ultrasonic bath for 30 min. The material was recovered and treated as described for the methylation analysis.

Acknowledgement

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