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Conformational stabilization of the altruronic acid residue in the O-specific polysaccharide of *Shigella sonnei/Plesiomonas shigelloides*

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Abstract

Complete assignments for the ^{1}H- and the ^{13}C-NMR spectra of the O-specific polysaccharide of *S. sonnei/Plesiomonas shigelloides* are reported. Evidence is presented that in this polysaccharide both pyranose residues exist preferentially in the $^{4}C_{1}$ chair conformation and that the polysaccharide exists in the zwitterion form. © 1998 Elsevier Science Ltd.

Keywords: Altruronic acid; NMR spectroscopy; *Plesiomonas shigelloides*; Polysaccharide conformation; *Shigella sonnei*; O-Specific polysaccharide

1. Introduction

Shigella causes dysentery, a severe infection of the large intestine, worldwide, especially in developing countries. *Shigella sonnei* is the most prevalent in developed countries [1–3]. The Phase 1 variant of this organism fully expresses its cell-surface lipopolysaccharide (LPS), a structurally distinct outer component of which is the O-specific polysaccharide (O-SP). The O-SP defines serogroup specificity and its full expression is an essential virulence factor [4]. Chemical and spectroscopic studies by Romanowska and Reinhold [5], Kontrohr [6], and Kenne et al. [7] showed that the O-SP of *S. sonnei* is a linear heteropolysaccharide (**1**) consisting of α-(1 → 3)-linked 2-acetamido-2-deoxy-L-altropyranuronic acid and β-(1 → 4)-linked 2-acetamido-4-amino-2,4,6-trideoxy-D-galactopyranose residues (Fig. 1), the latter of which was also shown to be present in the capsular polysaccharides of *Bacteroides fragilis* [8] and *Streptococcus pneumoniae* type 1 [9]. Immunochemical studies [10] and comparative spectroscopy [11] provided evidence that the O-SP of *Plesiomonas shigelloides* is identical to that of *S. sonnei*.

We have recently embarked on a program to synthesize defined fragments of the O-SP **1** for incorporation into neoglycoproteins that will eventually be evaluated as potential semi-synthetic vaccines for the prevention of dysentery in humans [12]. In connection with our interest in relating the NMR parameters of the synthetic saccharides with those of **1**, we were

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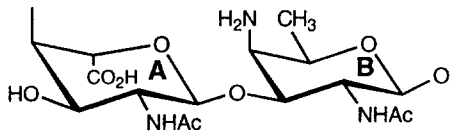


1

Fig. 1. The repeating unit of the O-specific polysaccharide of *S. sonnei / P. shigelloides*.

surprised to find out that a complete assignment of the NMR spectra of **1** is still missing and that during the structural elucidation only three NMR resonances of **1** had been previously identified, reflecting the then available techniques [7]. These are the signals belonging to H-5, C-4, and -5 of the trideoxygalactose unit. We now report NMR studies of polysaccharide **1** at 500 MHz. Because *S. sonnei* reverts in culture to its rough form devoid of O-SP, the O-SP from *Plesiomonas shigelloides* was used in the experiments described here. It was prepared by acid hydrolysis of its LPS to give a sample that had an average molecular weight around 25 kDa [11].

2. Results and discussion

The ^{1}H-NMR spectrum of **1** (Fig. 2), recorded at 305 K to position the HOD signal away from the saccharide protons, reflects a high degree of purity. The minor peaks are due to residual core monosaccharide units that survived the acid hydrolysis during removal of the Lipid A moiety, and remained attached to the O-SP through its reducing end. Noteworthy is that no trace of Kdo can be seen. The large coupling constants (8.2 and 8.5 Hz) of the anomeric protons confirmed the presence of 1,2-*trans* diaxial

Table 1
^{1}H chemical shifts[a] (δ) of the O-specific polysaccharide of *Shigella sonnei / Plesiomonas shigelloides* (**1**) and compounds **3–6**

Proton	Compound					
	1[b,c]		**3**	**4**	**5**[b]	**6**[d]
	Residue **A**	Residue **B**				
H-1	4.803	4.746	4.725	4.719	4.368	4.358
H-2	3.836	3.89	4.053	4.013	3.679	3.806
H-3	3.744	4.190	3.812	3.700	3.991	3.764
H-4	4.460	3.88	4.222	4.252	3.522	3.008
H-5	4.530	4.081	4.566	4.325	3.980	3.847
H-6	–	1.33	–	–	1.275	1.291
CH_3CO	1.998	2.041	2.015	2.010	1.969	2.042

[a]Determined at 500 MHz at 305K in D_2O.
[b]pD = 5.5.
[c]For labeling of the residues, see formula **1**.
[d]pD = 9.8.

pairs of protons in the component monosaccharides [7]. The identity of the doublet at 4.746 ppm as due to the anomeric proton (H-1_B) of the trideoxygalactose unit (residue B) was resolved by its cross-peak in the two-dimensional TOCSY spectrum with the CH_3 resonance at 1.346 ppm that must be a part of the spin system of that residue (Table 1). Other cross-peaks identified the remaining resonances for the trideoxygalactose unit. The assignment of the peak at 3.880 as H-4_B was based on a weak cross-peak with H-5_B, reflecting a small coupling (ca. 1 Hz) between these protons. The second anomeric resonance was assigned as H-1_A. By way of the COSY experiment, all resonances belonging to the altropyranuronic acid residue could be assigned without ambiguity (Table 1). The three-bond, H–H coupling con-

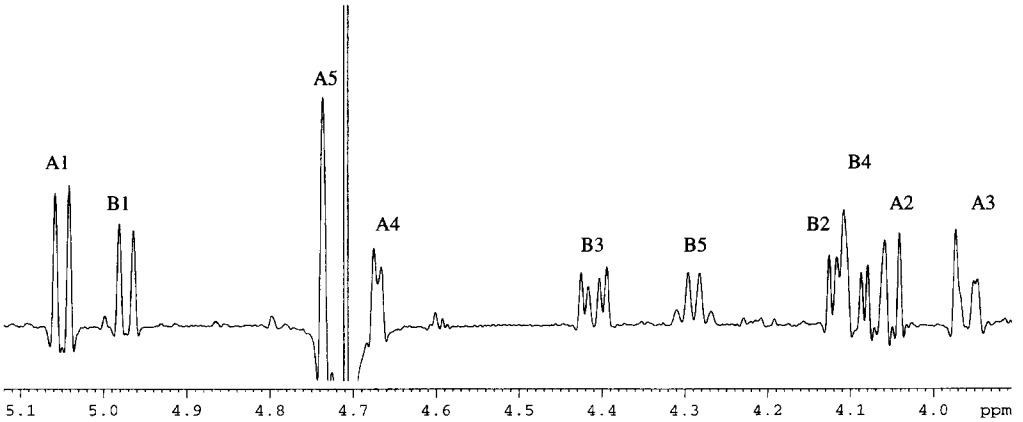


Fig. 2. Partial ^{1}H-NMR spectrum of the O-specific polysaccharide **1**. For the recording conditions, see the experimental section.

Table 2
Three-bond homonuclear H–H coupling constants (Hz) for the O-specific polysaccharide of *Shigella sonnei/Plesiomonas shigelloides* (**1**) and compounds **3–6**

Coupling constant	Compound					
	1[a]		**3**[b]	**4**	**5**[c]	**6**[d]
	Residue **A**	Residue **B**				
$J_{1,2}$	8.5	8.2	5.6 (8.6)	6.7	8.6	7.9
$J_{2,3}$	10.7	11.1	8.7 (10.6)	9.0	10.8	10.7
$J_{3,4}$	4.5	4.4	3.2 (2.7)	3.2	4.6	3.7
$J_{4,5}$	~ 1	nd	5.4 (1.9)	4.3	1.6	1.5
$J_{5,6}$	–	6.9	–	–	6.5	6.5

[a]For labeling of the residues, see formula **1**.
[b]The theoretical coupling constants for the optimized $^4C_1$ conformation, calculated by the program PCMODEL [25], are given in parentheses.
[c]pD = 5.5.
[d]pD = 9.8.

stants indicate that both pyranose rings in **1** exist preferentially in the $^4C_1$ conformation (Table 2).

The proton-detected ^1H–^{13}C correlation map unambiguously identified all the sugar carbons for which the one-bond, ^1H–^{13}C coupling constants were measured by the sensitivity-enhanced HSQC method without decoupling during acquisition [13,14] (Table 3). The PFG–HMBC spectra [15,16] confirmed the ^{13}C assignments and provided direct evidence about the position of the glycosidic linkages. It is proved that the trideoxygalactopyranosyl moiety (unit B) is linked to O-4 of the altruronic acid residue (cross-peaks H-1_B–C-4_A and C-1_B–H-4_A) which in turn is attached to O-3 of the trideoxygalactosyl residue (cross-peaks H-1_A–C-3_B and C-1_A–H-3_B).

Two-dimensional NOESY and off-resonance ROESY experiments (tilted spin lock axis, $q = 56°$) [17–19] were also performed to lend further support to the above-presented assignments (Fig. 3). These experiments indicated a number of intra-residual contacts that are in agreement with the proposed assignments (Table 4). The *N*-acetyl group at 2.041 ppm could be assigned as a part of residue **B**. Interestingly, the *N*-acetyl group of ring **A** at 1.998 ppm exhibits negligible NOE–ROE connectivities to other protons. Substantial inter-residual contacts were also

Table 3
^{13}C chemical shifts[a] (δ) of the O-specific polysaccharide of the *Shigella sonnei/Plesiomonas shigelloides* (**1**) and compounds **3–6** and one-bond heteronuclear ^1H–^{13}C coupling constants[b] (Hz)

Carbon atom	Compound					
	1[c]		**3**	**4**	**5**[d]	**6**[e]
	Residue **A**	Residue **B**				
C-1	101.88 (166)	103.84 (166)	101.26 (167)	101.05 (165)	103.20	103.40 (162)
C-2	52.33 (145)	51.70 (139)	52.80	52.76	52.78	52.36
C-3	68.56 (143)	76.45 (146)	69.62	69.96	68.66	72.18
C-4	78.36 (150)	55.69 (151)	68.46	69.69	55.56	57.89
C-5	78.36 (150)	68.33 (142)	73.85	76.73	68.38	71.40
C-6	–	16.31	–	–	16.41	16.67
CH_3CO	23.05	23.18	23.18	23.24	23.00	22.93
CH_3CO	175.23	175.23	175.57	175.69	175.91	nd
CO_2	174.76	–	174.26	176.72	182.14	–

[a]Determined at 125 MHz at 305K in D_2O, reference: internal 1,4-dioxane, $\delta = 67.40$ ppm.
[b]In parentheses.
[c]For labeling of the residues, see formula **1**.
[d]pD = 5.5.
[e]pD = 9.8.

seen. For example, strong cross-peaks were observed between H-1_A and H-3_B as well as H-1_B and H-4_A that require close proximity of the corresponding protons across the interglycosidic linkages. Furthermore, the weak cross-peak between H-5_A and H-$5_B/6_B$ indicates that these protons may be in close proximity. An off-resonance ROESY experiment at elevated temperature (323 K) showed an almost identical cross-peak pattern to that obtained at 305 K and the proton chemical-shift changes were less than a

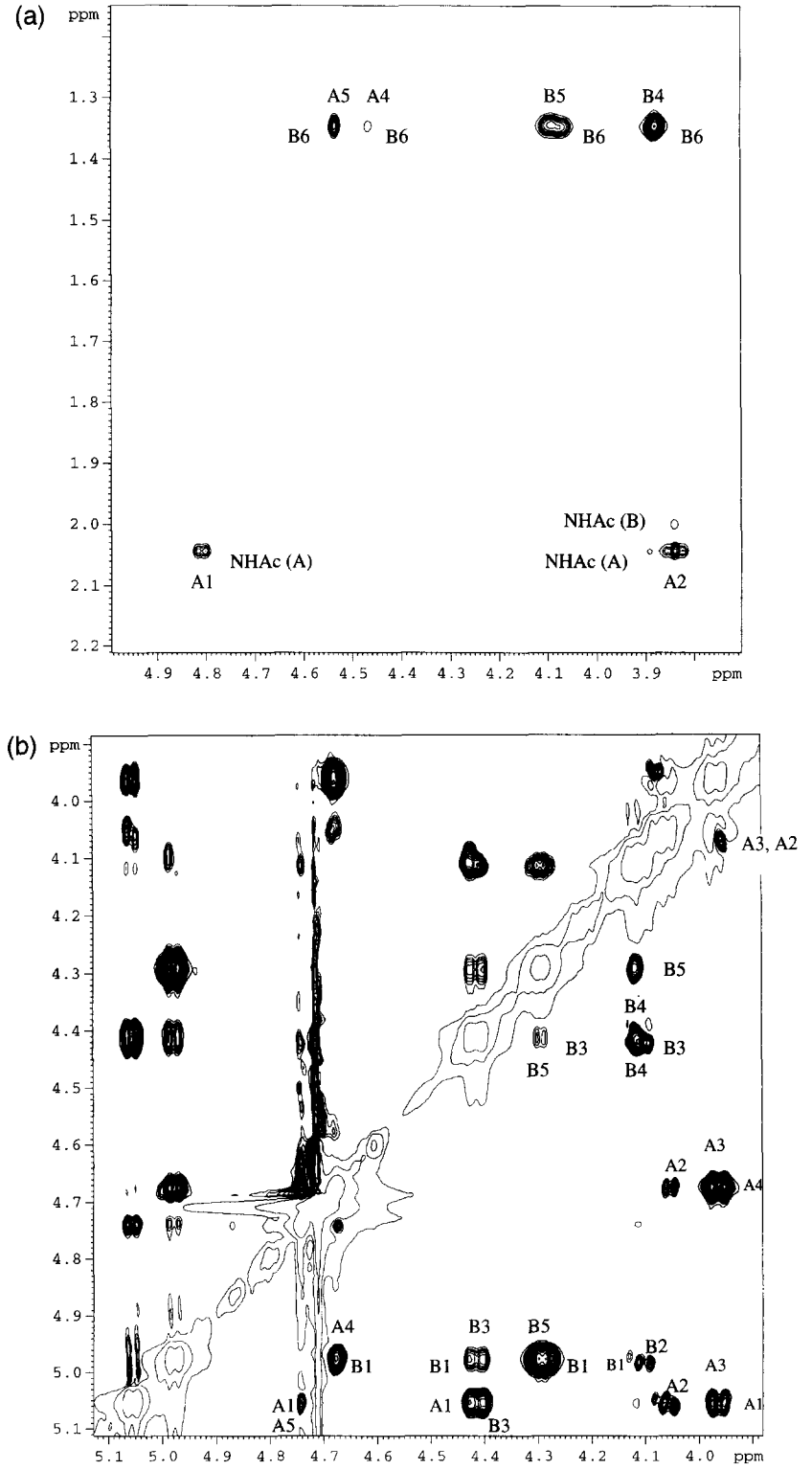


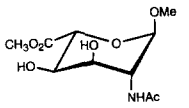
Fig. 3. Off-resonance ROESY spectrum of the O-specific polysaccharide **1**. The experimental conditions are detailed in the experimental section.

Table 4
Cross-peaks observed in the NOESY/off-resonance ROESY spectra of polysaccharide **1**

Proton[a]	Intra-residue cross-peak with	Inter-residue cross-peak with
H-1(A)	H-2, H-3, H-5	H-3(B)
H-4(A)	H-3, H-5	H-1(B)
H-1(B)	H-2, H-3, H-5	H-4(A)
H-2(A)	H-1, H-3	
H-6(B)	H-4, H-5	H-4(A), H-5(A)
H-4(B)	H-3, H-5, H-6	
H-5(A)	H-4, H-1	H-6(B)

[a]A = 2-Acetamido-2-deoxy-α-L-altropyranuronic acid, B = 2-Acetamido-4-2,4,6-trideoxy-β-D-galactopyranose.

few ppb. This observation suggests a remarkable stability of the tertiary structure of polysaccharide **1** in solution.



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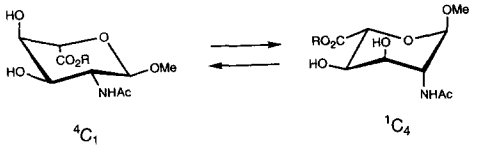
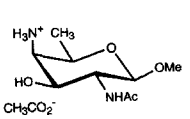


3 R = H
4 R = Na



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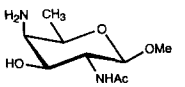

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Comparison of the NMR spectra of the trideoxy-galactose moiety (Residue **B**) in **1** with those of the protonated monosaccharide **5** (pD 5.45 [1]) shows the expected chemical shift differences, including a large downfield shift for C-3 and small upfield shifts for C-2 and -4 (Tables 1 and 3). This, and the remarkable similarity of the three-bond, homonuclear coupling constants, are in agreement with the protonation of the amino group in **1** (Table 2). Furthermore, the similarity of the coupling constants require the ring conformation of residue **B** to be identical to that of the $^4C_1$ chair form of monosaccharide **5**. On the other hand, the chemical shifts and the coupling constants of the free amine [12] **6** (pD 8.75) differ considerably from those of residue **B** in **1**, thus providing an indirect support for the protonation of the amino group in **1**. Inspection of the ^{1}H- and ^{13}C-NMR spectra of the 2-acetamido-2-deoxy-L-altruronic acid derivatives (**3**, **4**) that were obtained from the known methyl ester **2** [12] shows that the changes caused by the $CO_2H \rightarrow CO_2Na$ conversion are generally similar to those reported for the methyl glycoside of D-galactopyranuronic acid [20]. Notable are the large upfield shifts of H-5 (0.24 ppm) and C-5 atoms (2.88 ppm) in **4**. Surprisingly, significant differences are seen for the H-1–H-2 and for the H-4–H-5 couplings between compounds **3** and **4**, that are indicative of conformational differences (Table 2). Our calculations, using Haasnoot's formula [21] shows that the three-bond, homonuclear coupling constants in **3** are consistent with a 65:35 equilibrium between the $^4C_1$ and the $^1C_4$ conformers. In **4** the equilibrium is shifted toward the $^4C_1$ conformer with a small population of $^1C_4$ still being present as reflected by the $^3J_{1,2}$ (6.7 Hz) and $^3J_{4,5}$ (4.3 Hz) coupling constants. Since the methyl ester **2** was shown to exist almost exclusively in the $^1C_4$ chair conformation [12] we conclude that the conformations of the acetamido-altruronic acid derivatives **3** and **4** are mainly dictated by the charge at C-5 and that an increase of charge around this atom favors the $^4C_1$ conformer. For the proton pairs H-1–H-2 and H-2–H-3 in residue **A** of polysaccharide **1** there is a complete agreement between the experimental $^3J_{H,H}$ coupling constants and those calculated for the $^4C_1$ conformer. The slight difference for the H-3–H-4 and H-4–H-5 pairs is diagnostic of a minor deviation from the ideal $^4C_1$ chair shape for this part of residue **A**. These data strongly suggest that in polysaccharide **1** residue **A** adopts a single conformation. Since the monomeric 2-acetamido-2-deoxy-L-altruronic acid exists as a ca. 2:1 mixture of the $^4C_1$ and $^1C_4$ chair conformations, we conclude that its incorporation in polysaccharide **1** stabilizes the $^4C_1$ shape for this moiety.

3. Experimental

The O-specific polysaccharide of *Plesiomonas shigelloides* was obtained as described [11]. Optical

[1] The term pD as used in this paper refers directly to the pH meter reading without any correction that is discussed in Ref. [24].

rotations were measured at room temperature with a Perkin–Elmer 341 automatic polarimeter. Low resolution mass spectra were obtained by the chemical ionization technique (CIMS) using ammonia as the ionizing gas. Elemental analyses were performed by Atlantic Microlab, Norcross, GA. The NMR spectra were recorded on a Bruker DRX instrument operating at 500.13 MHz for protons and 125.78 MHz for carbons, equipped with a 5 mm inverse broadband probe. The samples were freeze-dried thrice from 99.9% D_2O before dissolution in '100%' D_2O obtained from Cambridge Isotope Laboratories. Chemical shifts (δ) are expressed in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate [22] for protons, but reference was made to internal sodium 4,4-dimethyl-4-sila-2,2,3,3-tetradeuteriopropionate (δ −0.018 ppm) or to internal acetone (δ 2.225 ppm). The ^{13}C-NMR spectra are referenced to internal 1,4-dioxane (67.40 ppm) [23], but the actual reference was made either to internal acetone (31.05 ppm) or to internal sodium 4,4-dimethyl-4-sila-2,2,3,3-tetradeuteriopropionate for which the $(CH_3)_3Si$ signal was set to −2.052 ppm to keep the $(CH_3)_2$ signal of acetone at 31.05 ppm. The 1D ^{1}H-NMR spectra were acquired by use of 64K data points. A spectral width of 2.5 kHz was employed, together with a 45 degree pulse (5 μs). The spectra were resolution-enhanced by Gaussian filtering (GB = 0.3) using a line broadening factor of −5. The 2D TOCSY spectrum was measured in the phase-sensitive mode using Bruker's standard pulse sequence by use of a spectral width of 2185 Hz, a 90 degree pulse width of 10 μs, and TD ($t2$) = 1024, and TD1 ($t1$) = 512 data points. 32 scans were used per $t1$ value with a mixing time of 60 ms. The data were processed in the phase sensitive time proportional phase increment (tppi) mode. The off-resonance ROESY experiment was carried out at 305 K in the phase-sensitive mode using the tppi method. The carrier frequency was placed at 5000 Hz. The spectral width was 2185 Hz in each dimension. The power of the spin-lock pulse was 10 dB below 50 W pulse power. The mixing time was 100 ms and 1024 data points were recorded in 512 increments; the acquisition time was 0.23 s. After zero filling the final resolution was 2.1 Hz/point. 32 scans were accumulated per $t1$ increment. The sensitivity-enhanced HSQC spectra were recorded with the standard pulse sequence supplied by Bruker with and without decoupling during acquisition. The ^{13}C carrier frequency was centered at 60 ppm. The spectral width was 1001 Hz (1024 data points) in the ^{1}H domain and 10000 Hz (256 data points) in the ^{13}C domain. The final resolution was 31 Hz/point. 16 scans were accumulated per $t1$ value.

Sodium (methyl - 2 - acetamido - 2 - deoxy - α - L - altropyranosid)uronate (**4**).—Compound [12] **2** (12.5 mg, 47.5 μmol)) was treated with 0.1 M aqueous NaOH (1 mL) for 1 min at 20 °C. The solution was treated with Dowex 50XB-100 (H^+) resin for 5 min at 20 °C followed by filtration. Freeze-drying of the solution gave amorphous methyl 2-acetamido-2-deoxy-α-L-altropyranosiduronic acid (**3**, quantitive). For the NMR spectra of **3** see Tables 1–3. A solution of **3** in H_2O was treated with Amberlite IRP-64 (Na^+) resin at 20 °C for 5 min. The mixture was filtered and the filtrate freeze-dried to afford amorphous **4** (quantitative): $[\alpha]_D$ −30° (c = 0.2, H_2O); for the NMR spectra see Tables 1–3; CIMS: m/z 289 [(M + NH_3)$^+$]. Anal. Calcd for $C_9H_{14}O_7NNa$ (271.20): C, 39.86; H, 5.20. Found: C, 39.62; H, 5.08.

Acetic acid salt of methyl 2-acetamido-4-amino-2,4,6-trideoxy-β-D-galactopyranoside (**5**).—Compound [12] **6** (25 mg) in H_2O (2 mL) was treated with acetic acid (200 μL). The solution was freeze-dried to afford **5** as a syrup. ^{1}H-NMR spectroscopy (in D_2O) indicated a 1:1 molar ratio of acetic acid and the amino sugar in the residue. For the NMR spectra, see Tables 1–3.

Acknowledgements

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