Structures of two O-chain polysaccharides of *Citrobacter gillenii* O9a,9b lipopolysaccharide

A new homopolymer of 4-amino-4,6-dideoxy-ß-mannose (perosamine)

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Mild acid degradation of the lipopolysaccharide of *Citrobacter gillenii* O9a,9b released a polysaccharide (PS), which was found to consist of a single monosaccharide, 4-acetamido-4,6-dideoxy-ß-mannose (ß-Rha4NAc, N-acetyl-ß-perosamine). PS was studied by methylation analysis and 1H-NMR and 13C-NMR spectroscopy, using two-dimensional 1H,1H COSY, TOCSY, NOESY, and H-detected 1H,13C heteronuclear correlation experiments. It was found that PS includes two structurally different polysaccharides: an α1→2-linked homopolymer of N-acetyl-ß-perosamine [(→2)-α-D-Rha4NAc(1→, PS2) and a polysaccharide composed of tetrasaccharide repeating units (PS1) with the following structure: →3)-α-D-Rha4NAc(1→2)-α-D-Rha4NAc(1→→3)-α-D-Rha4NAc(1→ where the degree of O-acetylation of a 3-substituted Rha4NAc residue at position 2 is ≈70%. PS could be fractionated into PS1 and PS2 by gel-permeation chromatography on TSK HW-50S. Matrix-assisted laser desorption ionization MS data indicate sequential chain elongation of both PS1 and PS2 by a single sugar unit, with O-acetylation in PS1 beginning at a certain chain length. Anti-(*C. gillenii* O9a,9b) serum reacted with PS1 in double immunodiffusion and immunoblotting, whereas neither PS2 nor the lipopolysaccharide of *Vibrio cholerae* O1 with a structurally related O-chain polysaccharide were reactive.

**Keywords:** 4-acetamido-4,6-dideoxy-ß-mannose; *Citrobacter gillenii* lipopolysaccharide; O-antigen; polysaccharide structure.

Strains of genus *Citrobacter* are inhabitants of the intestinal tract and, accordingly, are present in sewage, surface waters, and food contaminated with faecal material. Outbreaks of febrile gastroenteritis associated with food contaminated with faecal material. Outbreaks of febrile gastroenteritis associated with *Citrobacter* strains may cause opportunistic infections, including urinary and respiratory tract infections, especially in the immunocompromised host, and are also associated with meningitis, brain abscesses, and neonatal sepsis [1,2]. Currently, strains of the genus *Citrobacter* are classified into 11 species [3] and 43 O-serogroups [1,4]. Serological heterogeneity of *Citrobacter* strains is defined by the diversity in structures of the cell-surface lipopolysaccharide (LPS) [1,5]. With the aim of creating a molecular basis for classification of strains and substantiating their serological cross-reactivity, structures of the O-chain polysaccharides of LPS (O-antigens) of more than 20 serologically different *Citrobacter* strains have been established [6–8]. Now we report structural studies of LPS from *C. gillenii* O9a,9b, which is distinguished by the presence of two structurally different polysaccharide chains. Strains of this serogroup are often isolated from patients [1].

**MATERIALS AND METHODS**

**Bacterial strain, isolation and degradation of LPS**

*Citrobacter gillenii* O9a,9b:48 (strain PCM 1537) came originally from the Czech National Collection of Type Cultures, Prague (IHE Be 65/57, Bonn 16824 [1,5,9]) and was obtained from the collection of the Institute of Immunology and Experimental Therapy. Bacteria were cultivated in Davis broth supplemented with casem hydrolysate and yeast extract (Difco) with aeration at 37 °C for 24 h; they were then harvested and freeze-dried. LPS was isolated by phenol/water extraction and purified by ultra-centrifugation [10]. The yield of LPS was 3.2% of dry bacterial mass. A portion of LPS (200 mg) was heated with 1% acetic acid (20 mL) for 3 h at 100 °C, and the carbohydrate-containing supernatant was fractionated on a column (1.6 × 100 cm) of Bio-Gel P4 (~400 mesh) in 0.05 M aqueous pyridinium acetate buffer, pH 5.6, at a flow rate of 4 mL h⁻¹. The yield of polysaccharide material was 34 mg. Alternatively, carbohydrate material from another
portion of LPS (200 mg), degraded as above to obtain the carbohydrate-containing supernatant, was fractionated on a column (1.6 × 100 cm) of TSK HW-50S in the same pyridinium acetate buffer at a flow rate of 8 mL·h⁻¹. The yields of fractions 1, 2 (PS1), 3 (PS2), and 4 were 2.6, 11.4, 18.0 and 16.8%, respectively.

Chemical methods

O-deacetylation of PS (30 mg) was carried out with aqueous 12% ammonia at room temperature overnight followed by gel-permeation chromatography on a column (1.6 × 80 cm) of TSK HW-40S in water.

For sugar analysis, PS (0.4 mg) was hydrolysed with 10 M HCl for 30 min at 80 °C, and the alditol acetates derived were analysed by GLC-MS using a Hewlett-Packard 5971A system with an HP-1 glass capillary column (0.2 mm × 12 m) and temperature program of 8 °C·min⁻¹ from 150 to 270 °C. For determination of the absolute configuration [11,12], LPS (0.8 mg) was subjected to 2-butanolysis [300 μL (R)-2-butanol and 20 μL acetyl chloride, 100 °C, 3 h]; the products were acetylated and analysed by GLC-MS as above.

Methylation of PS (0.4 mg) was performed by the Hakomori procedure [13]; products were recovered by extraction with chloroform/water (1 : 1, v/v), hydrolysed with 10 M HCl for 30 min at 80 °C, and the partially methylated alditol acetates derived were analysed by GLC-MS as above.

NMR spectroscopy

Samples were freeze-dried twice from a 3H₂O solution and dissolved in 99.96% 3H₂O. 1H-NMR and 13C-NMR spectra were recorded with a Bruker DRX-500 spectrometer at 60 °C; chemical shifts are reported with internal acetone (δH 2.225, δC 31.45) as reference. Two-dimensional experiments were performed using standard Bruker software. A mixing time of 200 ms was used in TOCSY and HMQC-TOCSY experiments and 300 ms in a NOESY experiment.

Matrix-assisted laser desorption ionization (MALDI) MS

MALDI mass spectra were recorded on a RETOF (time-of-flight) instrument from Perseptive Biosystems (Framingham, MA, USA) equipped with a pulsed delay source extractor [14]. Spectra were recorded from 256 laser shots (nitrogen laser, 337 nm) with an accelerating voltage of 20 kV in linear mode. For a matrix, 2,5-dihydroxybenzoic acid was dissolved in aqueous 70% acetonitrile containing 0.1% trifluoroacetic acid. Then 1 μL acid was dissolved in aqueous 70% acetonitrile containing 0.1% trifluoroacetic acid. Then 1 μL of PS was mixed with 1 μL of sulfolane, placed on top of the matrix surface, and allowed to dry by itself. The spectra were calibrated using insulin (1 pmol μL⁻¹; m/z 5736) in the same conditions. Mass numbers were rounded to the nearest integer.

Rabbit antiserum, antigens and serological techniques

Rabbit antiserum against whole cells of C. gillenii O9a,9b was prepared as described previously [15]. LPS of Hafnia alvei PCM 1186 was from previous studies [15]. LPS of V. cholerae O1 was a gift from O. Holst (Forschungszentrum Borstel, Germany), and that of Escherichia coli O157 was a gift from B. Maćzyńska and A. Przondo-Mordarska (Medical Academy, Wrocław, Poland). SDS/PAGE and immunoblotting with LPS and double immunodiffusion with LPS and polysaccharides were performed as described previously [15–17].

RESULTS AND DISCUSSION

A high-molecular-mass PS was isolated by mild acid degradation of LPS of C. gillenii O9a,9b followed by gel-permeation chromatography of the carbohydrate portion on Bio-Gel P-4. Sugar analysis of PS revealed a 4-amino-4,6-dideoxyhexose as the single monosaccharide constituent. This was identified as 4-amino-4,6-dideoxy-o-mannose (o-Rha4N) as well as 4-amino-4,6-dideoxy-o-p-mannose (p-Rha4N), by comparison with the corresponding authentic samples from LPS of V. cholerae O1 [18] using GLC-MS of the alditol acetates and acetylated (R)-2-butyglycosides.

Methylation analysis of PS revealed 4,6-dideoxy-3-O-methyl-4(4-N-acetylacetamidomannose and 4,6-dideoxy-2-O-methyl-4(4-N-methyl)acetamidomannose in the ratio of 2 : 1, which were identified by GLC-MS of partially methylated alditol acetates (retention times 8.98 and 9.03 min, respectively). The former compound was characterized by the presence in the mass spectrum of intense ion peaks for the C1–C3, C1–C4, and C4–C6 primary fragments at m/z 190, 275, and 172, respectively. The mass spectrum of the latter compound showed intense ion peaks for the fragments C1–C2, C1–C4, and C4–C6 at m/z 118, 275, and 172, respectively. Hence, PS is linear and contains 2-substituted and 3-substituted perosamine residues. Further studies showed that PS includes two polysaccharides with the same sugar composition but different structures.

The 13C-NMR spectrum of PS (Fig. 1, top) contained signals with different integral intensities that could be due to nonstoichiometric O-acetylation (there was a signal for CH₃COO at δ21.5). Some minor signals could belong to the LPS core constituents as they were still present after O-deacetylation of PS with aqueous ammonia. The 13C-NMR spectrum of the O-deacetylated polysaccharide (PSNH₄OH, Fig. 1, bottom) was less complex than the spectrum of the initial PS and contained signals for several different Rha4NAc residues including signals for anomeric carbons (C1) at δ101.6–102.9, carbons bearing nitrogen (C4) at δ52.9–54.3, CH₃-C groups (C6) at δ18.0–18.3, and N-acetyl groups at δ23.3–23.5 (CH₃) and 175.0–175.7 (CO).

In each carbon group, some signals were two to five times as intense as the single signal. According to the 3H-NMR spectrum of PSNH₄OH (Table 1), one of the signals contained signals for several different Rha4NAc residues including signals for anomic protons (H1) at δ4.96–5.13, CH₃-C groups (H6) at δ1.17–1.22, and N-acetyl groups at δ2.04. The two-dimensional COSY and TOCSY spectra of PSNH₄OH revealed spin systems for five different Rha4NAc residues, all signals for one of them (Rha4NAcο) being about twice as intense as signals for each of four other residues (Rha4NAc₁, Rha4NAc₁). At the H1 co-ordinate, the TOCSY spectrum showed cross-peaks with H2–H6 for Rha4NAcο–Rha4NAcο and only two cross-peaks, with H2 and H3, for Rha4NAcο. At the H6 co-ordinate, the spectrum showed cross-peaks for the whole spin system of each monosaccharide residue. The COSY spectrum allowed differentiation into different structures.
Difficulties associated with coincidence of signals for some neighboring protons (H3 and H4 of Rha4NAcI and Rha4NAcII) were overcome using an H-detected $^1$H,$^1$3C heteronuclear single-quantum coherence (HSQC) experiment. This also confirmed the assignment for H4 by their correlation to C4 located in the resonance region of carbons bearing nitrogen ($\delta$ 52.9–54.3).

The $^1$3C-NMR spectrum of PSNH4OH (Table 2) was assigned using a $^1$H,$^1$3C HSQC experiment. The assignment for C2 was additionally confirmed by a combined $^1$H,$^1$3C HMQC-TOCSY experiment (Fig. 2), which revealed clear correlation between H1 and C2. Chemical shifts for C5 ($\delta$ 69.3–69.6) in the $^1$3C-NMR spectra of PSNH4OH and an $\alpha$1→2-linked $\alpha$-Rha4NAc homopolymer from V. cholerae bio-serogroup Hakata [19] (serogroup O140 [20]) were close and, hence, all Rha4NAc residues are $\alpha$-linked (C5 of $\beta$-pyranosides is known to resonate in a lower field than C5 of $\alpha$-pyranosides [21]). The relatively low-field position at $\delta$ 78.0–79.3 of the signals for C3 of Rha4NAcII and Rha4NAcV and C2 of three other Rha4NAc demonstrated the mode of substitution of the monosaccharides (compare the position at $\delta$ 69.0–70.6 of the signals for nonlinked C2 and C3 of Rha4NAc; Table 2).

A NOESY experiment (Fig. 3) revealed strong intraresidue H1/H2 correlations for Rha4NAcI and Rha4NAcII at $\delta$ 5.13/4.12 and 4.97/3.85 and weaker H1/H2 correlations for Rha4NAcIII–Rha4NAcV (the latter are below the level shown in Fig. 3). Most importantly, the spectrum contained interresidue cross-peaks between the following transglycosidic protons: Rha4NAcII H1/Rha4NAcV H3 at $\delta$ 4.97/3.98, Rha4NAcV H1/Rha4NAcIV H2 at $\delta$ 5.03/4.13, Rha4NAcIV H1/Rha4NAcIII H2 at $\delta$ 5.10/3.79, and Rha4NAcIII H1/Rha4NAcII H3 at $\delta$ 4.96/3.91. These data are in agreement with the $^1$3C-NMR chemical-shift data and show a Rha4NAc homopolysaccharide with a tetrasaccharide repeating unit (PS1NH4OH; Fig. 4). No interresidue cross-peak was observed for Rha4NAcI but a strong intraresidue H1/H2 cross-peak at $\delta$ 5.13/4.12 and a weak H1/H5 cross-peak at $\delta$ 5.13/3.82 typical of $\alpha$1→2-linked sugars with the manno configuration. Hence, Rha4NAcI residues are $\alpha$1→2-linked and build another polysaccharide chain (PS2; Fig. 4).

Comparison of the $^1$H-NMR, $^1$3C-NMR, and $^1$H,$^1$3C HMQC spectra of PSNH4OH and PS enabled the determination of the site of attachment of the O-acetyl group. In the $^1$H,$^1$3C HMQC spectrum, the intensity of the H2/C2

### Table 1. $^1$H-NMR data.

<table>
<thead>
<tr>
<th>Sugar residue</th>
<th>Chemical shift (p.p.m.)</th>
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<tbody>
<tr>
<td>PS1</td>
<td>H1 3.72, H2 3.74, H3 3.78, H4 3.84, H5 3.87, H6 1.21</td>
</tr>
<tr>
<td>PS2</td>
<td>H1 4.10, H2 3.79, H3 3.95, H4 3.98, H5 3.97, H6 1.18</td>
</tr>
</tbody>
</table>

Fig. 1. 125-MHz $^1$3C-NMR spectra of the initial (PS, top) and O-deacetylated (PSNH4OH, bottom) polysaccharides from C. gillenii O9a,9b.
cross-peak of Rha4NAcII at $\delta$ 3.85/70.6 markedly decreased and a new cross-peak appeared at $\delta$ 5.00/72.1. The $^{13}$C-NMR spectrum displayed displacements of parts of the signals for C1 and C3 of Rha4NAcII from $\delta$ 102.9 and 78.0 to $\delta$ 101.6 and 76.6, respectively, which are typical of $\beta$-effects of acetylation at O2 [22]. Therefore, part of the Rha4NAcII residues is O-acetylated at position 2, and PS1 thus has the structure shown in Fig. 4. As judged by the ratio of the integral intensities of the signals for the O-acetylated and non-O-acetylated residues, the average degree of O-acetylation of Rha4NAcII in PS1 is $\approx$ 70%. PS2 contains no O-acetyl group.

To confirm the existence of two polysaccharides, the carbohydrate portion obtained after mild acid degradation of $C. gillenii$ O9a,9b LPS was fractionated by gel-permeation chromatography on TSK HW-50S to give six fractions (Fig. 5). The MALDI mass spectrum of fraction 1 revealed a series of hexose increments with $m/z$ 162, and this fraction was considered to be a glucan-type contaminant. Fraction 4 represented a core oligosaccharide, and fractions 5 and 6 contained low-molecular-mass compounds released from LPS. 1H-NMR and 13C-NMR spectroscopic analysis showed that the perosamine-containing polysaccharides PS1 and PS2 were present in fractions 2 and 3, respectively. Therefore, the two polysaccharides could be separated and thus belonged to separate LPS molecules. The MALDI mass spectrum of PS1 showed a series of ion peaks with differences between ions of 187 or 229 Da, which corresponded to non-O-acetylated and O-acetylated Rha4NAc, respectively (Fig. 6). The low-molecular-mass polysaccharide species (below 4258 Da) were devoid of O-acetyl groups. The difference between the ions at $m/z$ 4258 and 4487 corresponded to the O-acetylated Rha4NAc residue (Rha4NAc2AcII), and the next three peaks in this series at $m/z$ 4674, 4861 and 5048 reflected further chain elongation by non-O-acetylated residues (Rha4NAcIII–Rha4NAcV) to complete the tetrasaccharide repeating unit of PS1. Then, starting from the ion peak at $m/z$ 5048, the pattern iterated. The next ion peaks with a difference of 790 Da for the mono-O-acetylated tetrasaccharide (indicated by arrows), as well as the intermediate ion peaks (shown by asterisks), were clearly observed up to $m/z$ 7418. Some of the minor peaks may be due to heterogeneity of the core oligosaccharide. The 18 Da difference between ions (at 2949 and 2967 $m/z$ and the next peaks in this series) may result from the dehydrated and hydrated forms of 3-deoxy-octulosonic acid (Kdo) residue, respectively, at the reducing end of the polysaccharide.

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Table 2. $^{13}$C-NMR data. Additional chemical shifts for the N-acetyl groups are: $\delta$ 23.3–23.5 (CH$_3$) and 175.0–175.7 (CO).

<table>
<thead>
<tr>
<th>Sugar residue</th>
<th>C1</th>
<th>C2</th>
<th>C3</th>
<th>C4</th>
<th>C5</th>
<th>C6</th>
</tr>
</thead>
<tbody>
<tr>
<td>O-Decetylated PS1</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>$\rightarrow$ 3) $\alpha$-o-Rhap4NAcII-(1 $\rightarrow$</td>
<td>102.9</td>
<td>70.6</td>
<td>78.0</td>
<td>53.1</td>
<td>69.6</td>
<td>18.0</td>
</tr>
<tr>
<td>$\rightarrow$ 2) $\alpha$-o-Rhap4NAcIII-(1 $\rightarrow$</td>
<td>101.9</td>
<td>79.3</td>
<td>69.0</td>
<td>54.3</td>
<td>69.3</td>
<td>18.0</td>
</tr>
<tr>
<td>$\rightarrow$ 2) $\alpha$-o-Rhap4NAcIV-(1 $\rightarrow$</td>
<td>101.9</td>
<td>78.2</td>
<td>69.0</td>
<td>54.3</td>
<td>69.4</td>
<td>18.3</td>
</tr>
<tr>
<td>$\rightarrow$ 3) $\alpha$-o-Rhap4NAcV-(1 $\rightarrow$</td>
<td>102.7</td>
<td>70.1</td>
<td>78.2</td>
<td>52.9</td>
<td>69.6</td>
<td>18.0</td>
</tr>
<tr>
<td>PS2$^c$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\rightarrow$ 2) $\alpha$-o-Rhap4NAcI-(1 $\rightarrow$</td>
<td>101.6</td>
<td>78.2</td>
<td>69.0</td>
<td>54.3</td>
<td>69.6</td>
<td>18.0</td>
</tr>
<tr>
<td></td>
<td>(101.33)</td>
<td>(77.86)</td>
<td>(68.67)</td>
<td>(53.91)</td>
<td>(69.33)</td>
<td>(17.64)</td>
</tr>
</tbody>
</table>

$^a,b$ Assignment could be interchanged. $^c$ Data from [19] for the O-specific polysaccharide of $V. cholerae$ bio-serogroup Hakata (serogroup O140 [20]) are given in parentheses. The differences in the chemical shifts are due to the use of different references for calibration (dioxane in the published work [19] and acetone in this work).
The MALDI mass spectrum of PS2 (not shown) displayed a series of ion peaks with a difference between ions of 187 Da, which corresponded to sequential chain elongation by one non-O-acetylated Rha4NAc residue. The intensities of the first peaks for the short-chain polysaccharide species were high and those of the following peaks decreased, but the series could be traced up to 20 and more Rha4NAc residues.

The data obtained suggested that growth of both PS1 and PS2 in *C. gillenii* O9a,9b proceeds by sequential transfers of single sugar units. A biosynthetic model involving sequential single sugar transfers to the nonreducing end of the growing chain has been suggested for the A-band polysaccharide (D-rhamnan) in *Pseudomonas aeruginosa* LPS [23] as well as for linear homopolysaccharide O-antigens of *Escherichia coli* O8 and O9 (D-mannans) and D-galactan I from *Klebsiella pneumoniae* (reviewed in [24]). This model requires participation of several distinct transferases for the same monosaccharide, as demonstrated for biosynthesis of the A-band polysaccharide [23].

A polysaccharide with the same structure as PS2 has been previously reported to be the O-chain of the LPS of *V. cholerae* bio-serogroup Hakata [19] (serogroup O140 [20]), whereas PS1 is new. Interestingly, a polysaccharide of α1 → 2-linked and α1 → 3-linked 4-formamido-4,6-dideoxy-D-mannose (N-formyl-D-perosamine) having a pentasaccharide repeating unit has been found in *Brucella melitensis* LPS [25]. Published structural data [25] do not exclude the occurrence of two separate polysaccharide chains in the LPS of *B. melitensis*. The O-chain homopolymer from *Escherichia hermannii* LPS composed of α1 → 2-linked and α1 → 3-linked D-Rhap4NAc residues has been reported to have a pentasaccharide repeating unit containing the tetrasaccharide sequence present in PS1 [26].

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Fig. 3. Part of a NOESY spectrum of the O-deacetylated polysaccharide (PSNH4OH) from *C. gillenii* O9a,9b. The corresponding parts of the 1H-NMR spectrum are displayed along the axes. Arabic numerals refer to protons in sugar residues denoted by roman numerals as shown in Fig. 4.

Fig. 4. Structures of the polysaccharides (PS1 and PS2) and the O-deacetylated polysaccharide (PS1NH4OH) from *C. gillenii* O9a,9b.

Fig. 5. Fractionation on TSK HW-50S of the carbohydrate material obtained by mild acid hydrolysis of *C. gillenii* O9a,9b LPS. For explanation of fractions, see the text.

The MALDI mass spectrum of PS2 (not shown) displayed a series of ion peaks with a difference between ions of 187 Da, which corresponded to sequential chain elongation by one non-O-acetylated Rha4NAc residue. The intensities of the first peaks for the short-chain polysaccharide species were high and those of the following peaks decreased, but the series could be traced up to 20 and more Rha4NAc residues.
LPS of C. gillenii O9a,9b reacted with homologous anti-O serum in double immunodiffusion (data not shown). In SDS/PAGE and immunoblotting (Fig. 7), anti-(C. gillenii O9a,9b) serum reacted mainly with slowly moving, high-molecular-mass LPS species. O-Deacylation of C. gillenii O9a,9b LPS had no effect on its serological reactivity. From the separated O-chain polysaccharides, only PS1 reacted in double immunodiffusion with anti-(C. gillenii O9a,9b) serum, whereas PS2 was inactive, probably, because of a lower molecular mass.

No significant cross-reactivity was observed between anti-(C. gillenii O9a,9b) serum and V. cholerae O1 LPS in double immunodiffusion (not shown) and immunoblotting (Fig. 7). This can be accounted for by different N-acyl substituents at d-Rha4N: N-acetyl or N-[S]-2,4-dihydroxybutyryl] group in the O-antigens of C. gillenii and V. cholerae [18], respectively. The LPS from E. coli O157, which also contains d-Rha4N [24], also did not react with anti-(C. gillenii O9a,9b) serum in double immunodiffusion (data not shown).

ACKNOWLEDGEMENTS
We thank Professor O. Holst (Forschungszentrum Borstel, Germany) for the gift of V. cholerae O1 LPS and Dr B. Maćzyńska and Professor A. Przondo-Mordarska (Medical Academy, Wrocław, Poland) for the gift of E. coli O157 LPS. This work was supported by grant 99-04-48279 from the Russian Foundation for Basic Research and grant 500-1-15 from the Polish Academy of Sciences.

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