Structural Determination of the *O*-Specific Polysaccharide from the *Xanthomonas fragariae* Lipopolysaccharide Fraction

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Keywords: *Xanthomonas fragariae* / Natural products / Structure elucidation / Bioorganic chemistry / Lipopolysaccharides

The structure of the *O*-specific polysaccharide from the lipopolysaccharide fraction of the phytopathogenic bacterium *Xanthomonas fragariae*, the causal agent of the angular leaf spot of strawberry, is described. On the basis of the spectroscopic data, chemical analyses and degradation methods, a structure is suggested.

Introduction

Lipopolysaccharides (LPSs) are the most abundant macromolecules present in the outer membrane of Gram-negative bacteria[1] and they are involved in many interactions of the cells with the external environment. In particular, in the case of phytopathogenic bacteria the role of LPS in the virulence factors has been claimed.[2]

Recent investigations have shown that the strawberry plant is infected by *Xanthomonas fragariae*, the causal agent of the angular leaf spot of strawberry, and by another xanthomonad which, on the basis of the pathogenic and biochemical features, may be ascribed to *X. campestris.*[3] Although the symptoms of the two diseases are different a misinterpretation of the causal agents is possible; in fact mixed infections have been reported to occur. The recognised economic importance of the angular spot disease of strawberry prompted us to perform a comparative polyphasic characterisation of the two phytopathogenic bacteria. Here, after the structural characterisation of the *Xanthomonas campestris O*-chain,[4] the chemical characterization of the *O*-chain from *Xanthomonas fragariae* is reported.

Results and Discussion

The lipopolysaccharide fraction of *Xanthomonas fragariae* was obtained from the aqueous phase of phenol/water treatment of dried cells. The electrophoresis behaviour on SDS-PAGE, KDO (3-deoxy-D-*manno*-octulosonic acid) assay and fatty acids analysis indicated the LPS nature of the extract material, which contained only traces of protein. Mild acid hydrolysis of the LPS fraction yielded the lipid moiety, as a precipitate, and in the liquid phase the *O*-specific polysaccharide (OPS), which was purified by elution in the void volume on a Bio-Gel P-10 column. Further gel permeation chromatography (Bio-Gel P100, Bio-Gel A 0.5 m, Sephacryl S-300) showed the presence of a single peak in all cases, suggesting a homogeneous product whose average molecular weight was determined to be 30 kDa. The chemical analysis indicated a monosaccharide composition consisting only of fucose and rhamnose in about a 1:2.5 ratio. The absolute configurations of these sugars, on the basis of the GLC analysis of their 2-octyl glycoside acetates, was measured to be D for Fuc and L for Rha. The methylation analysis of OPS showed the presence of terminal-Fuc*p*, 3-linked Rha*p* and 2,3-linked Rha*p* in about a 1:1:1 ratio. The ^{1}H and ^{13}C NMR spectra (Figure 1 A and B) showed at high-field two methyl signals of 6-deoxysugars, occurring at $\delta = 1.21$ and 1.34, which were assigned to Fuc and Rha, respectively (vide infra), whereas at low fields they showed crowded anomeric and carbon glycosylate regions, which suggested a nonregular OPS structure. However, a thorough NMR investigation by one- and two-dimensional experiments (COSY, TOCSY, ROESY, HSQC, HMBC) allowed us to identify at least six main residues (A−F) whose relevant proton and carbon signals were partially assigned (Table 1). From these data the α-anomeric configuration of the Fuc units was inferred by the absence of anomeric carbon signals at field lower than $\delta = 102.8$, whereas Rha was present both in the α and β configurations.[5] The β configuration of the F residue was evident from the high field ^{1}H NMR spectrum of the anomeric proton ($\delta = 4.869$), and the downfield ^{13}C NMR resonance of C-5 ($\delta = 72.9$). In addition, the *syn*-diaxial orientation of H-1, H-3 and H-5, which was deduced by the intraresidue NOEs among these protons in the ROESY spectrum, confirmed the β configuration. Moreover, F appeared to be 2,3-linked since both C-2 and C-3 experienced glycosylation shifts at lower fields with respect to those of an unsubstituted β-Rha residue.[6] The data of Table 1 suggested further structural features. In particular, the A and D units were assigned to terminal Fuc residues because no carbon signals experienced glycosyl-

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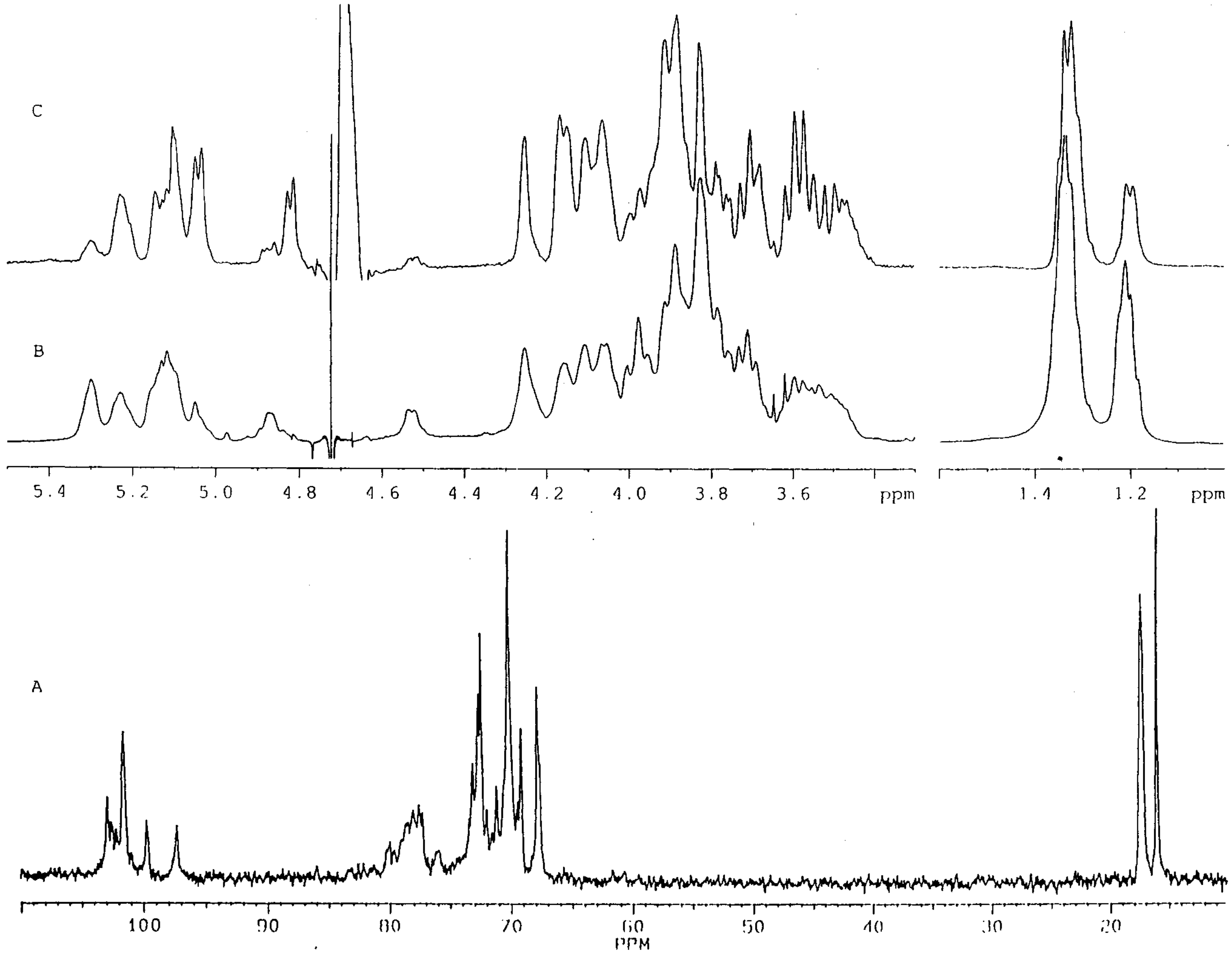


Figure 1. ^{13}C (A) and ^{1}H NMR (B) spectra of the *Xanthomonas fragariae* OPS and ^{1}H NMR (C) spectrum of the *Xanthomonas fragariae* HP (400 MHz, D_2O, 30 °C)

ation shifts. The B, C and E units were all attributed to α-Rha*p* residues considering both the low-field chemical shift values of their anomeric protons and the high field position of their C-5 signals (δ = 69.6−69.8).[6] Furthermore, the involvement of the positions 2 and 3 of the B unit and of the position 3 for C and E was deduced by the glycosylation shifts of their C-2 and C-3 signals. The HMBC experiment led us to measure H,C interresidue long-range coupling between the anomeric protons/carbons of the terminal-Fuc*p* A and D units and the carbons/protons of the atoms at position 2 of F (β-Rha*p*) and B (α-Rha*p*), respectively. The ROESY experiment supported the above conclusion.

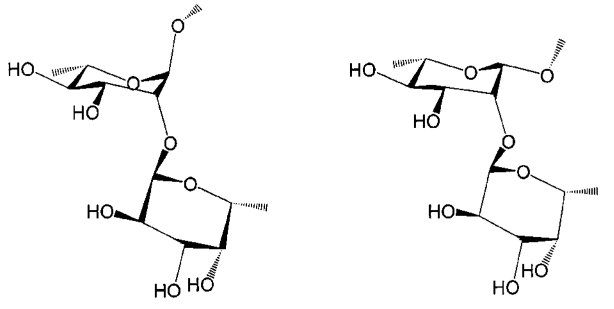
A mild acid hydrolysis performed on the OPS polysaccharide resulted in partial hydrolysis of fucose, but not rhamnose, giving a degraded polysaccharide (HP). Assignment of the methyl signal (Figure 1C) at δ = 1.21 to fucose was confirmed by the decrease in the intensity of that signal along with those at δ = 5.30 (anomeric proton, fucose unit A) and δ = 4.53 (H-5, fucose unit A). This allowed us to identify the fucose unit linked at β-Rha*p* as that released during the acid hydrolysis. Interestingly, such evidence indicated a hydrolysis rate of this fucose higher than that of fucose on α-Rha*p*. This might be due to the higher steric relief provided by the removal of the β-Rha*p* residue be-

Table 1. ^{1}H and ^{13}C NMR resonances and assignments of the *Xanthomonas fragariae* OPS (400/100 MHz, in D_2O at 30 °C); the assignments were made on the basis of two-dimensional experiments (COSY, TOCSY, ROESY, HSQC and HMBC)

Residue	$\delta_{H-1/C-1}$	$\delta_{H-2/C-2}$	$\delta_{H-3/C-3}$	$\delta_{H-4/C-4}$ [a]	$\delta_{H-5/C-5}$	$\delta_{H-6/C-6}$
Terminal-α-Fuc*p* **A**	5.30/99.5	3.80/69.5	3.98/69.9	3.53/71.0	4.53/67.4	1.21/15.9
2,3-α-Rha*p* **B**	5.23/101.3	4.11/77.8	3.97/77.3	3.94/72.0	3.82/69.7	1.34/17.3
3-α-Rha*p* **C**	5.14/101.8	4.26/70.0	3.97/76.9	n.d.	3.98/69.8	1.34/17.3
Terminal-α-Fuc*p* **D**	5.12/101.6	3.76/69.0	3.90/69.9	3.74/72.8	4.06/67.4	1.21/15.9
3-α-Rha*p* **E**	5.05/102.8	4.16/70.3	3.93/79.3	n.d.	3.84/69.6	1.34/17.3
2,3-β-Rha*p* **F**	4.87/97.2	4.25/75.7	3.67/80.3	n.d.	3.47/72.9	1.34/17.3

[a] n.d.: not detected.

cause its 2-position is more crowded than that of an α-Rha*p* unit, owing to the *gauche* orientation of the 2-oxygen with the glycosidic oxygen, as can be seen in the disaccharide models shown in Figure 2. Accordingly, the assignment of the proton and carbon signals of β-Rha*p*, obtained starting from the proton/carbon anomeric signals in HP occurring at δ = 4.82/97.4, indicated the high-field shift (δ = 71.4) of the carbon signal at position 2, now unsubstituted, whereas the C-3 chemical shift at δ = 80.8 indicated that this position was still glycosylated.



a) α-D-Fuc*p* (1-2)-α-L-Rha*p*-OCH_3 b) α-D-Fuc*p* (1-2)-β-L-Rha*p*-OCH_3

Figure 2. Three-dimensional drawing of two disaccharide models showing the *anti* (a) and the *gauche* (b) orientation of the oxygen at C-2 with the glycosidic oxygen.

A Smith degradation of the *O*-chain polysaccharide, followed by Bio-Gel chromatography, gave the backbone (SP) of OPS consisting of α and β 3-linked Rha*p* units. The α/β ratio of the rhamnose residues was determined from the ^1H and ^{13}C NMR spectra (Figure 3) both by integration of the proton anomeric signals and by integration of the anomeric and glycosylate carbon signals in a ^{13}C inverse-gated-decoupled spectrum.[7] In all cases an average value of 3.5 was estimated, suggesting a 7:2 ratio of α/β Rha*p* residues, respectively. However, from a detailed NMR analysis we were able to obtain the complete proton and carbon signal assignment for only four types of residues (A′−D′) (Table 2). Therefore the chemical shift values of some units must be coincidental.

The HMBC spectrum of SP shows a scalar long-range coupling between the proton anomeric signal of the β-Rha*p* D′ unit (δ = 4.83) and C-3 (δ = 78.2) of the A′ unit, between the anomeric proton of this latter (δ = 5.11) and C-3 of B′ (δ = 79.2) and between the anomeric proton of C′ (δ = 5.04) and C-3 of D′ (δ = 81.2) allowing us to establish the sequence C′(1→3)D′(1→3)A′(1→3)B′. The other distinguishable anomeric proton of SP at δ = 5.05 of the B′ unit was long-range correlated with a C-3 whose signal occurred at δ = 79.2. This value is the same both for the B′ and C′ units, therefore two possible sequences should be conceivable: the first, B′(1→3)C′, should imply a tetrasaccharide repeating unit with an α/β Rha ratio of 3:1, whereas the second, B′(1→3)B′, should imply an α/β Rha ratio of 4:1. Since we found an average value of 3.5, we suggested for the backbone a structure constituted of a combination of the two sequences [→3)C′(1→3)D′(1→3)A′(1→3)-

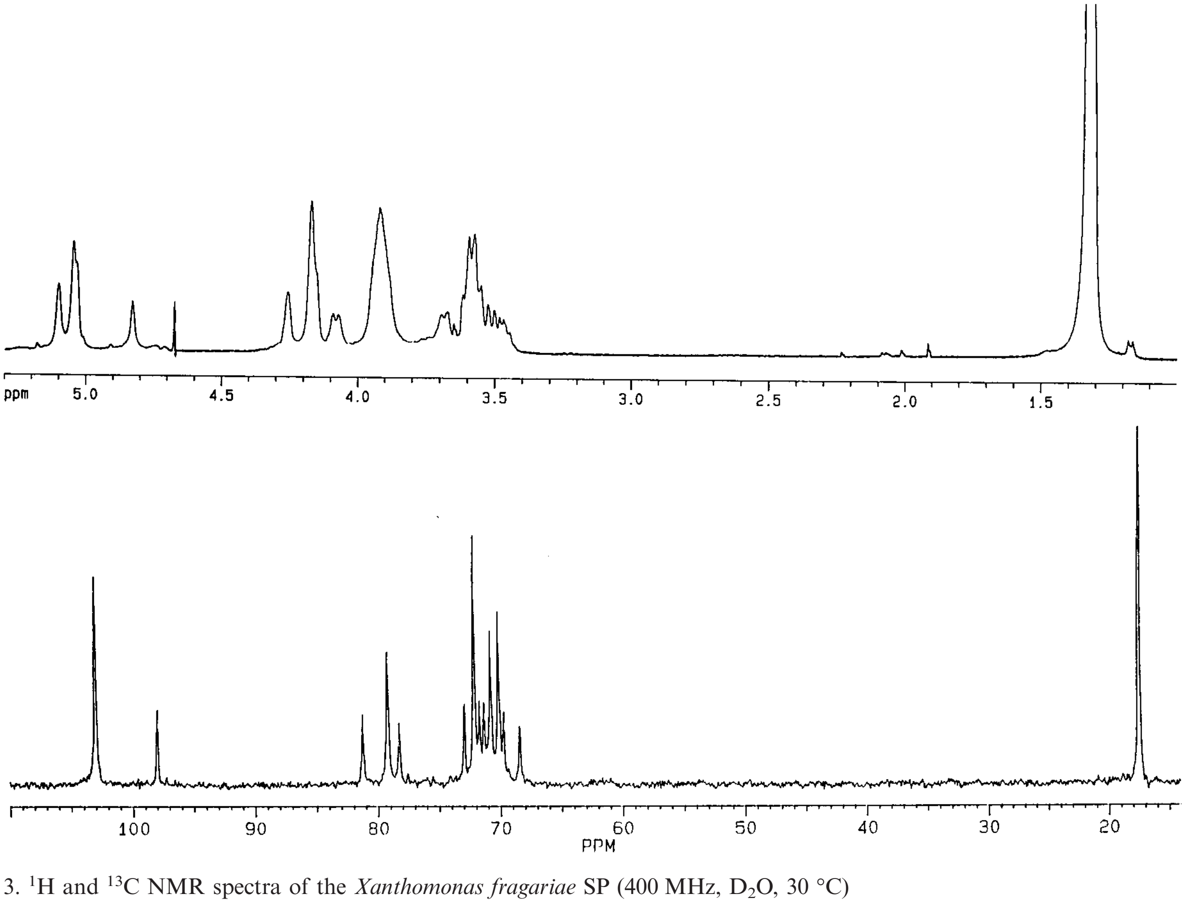


Figure 3. ^1H and ^{13}C NMR spectra of the *Xanthomonas fragariae* SP (400 MHz, D_2O, 30 °C)

Table 2. ^{1}H and ^{13}C NMR resonances and assignments of the *Xanthomonas fragariae* SP (400/100 MHz, in D_2O at 30 °C); the assignments were made on the basis of two-dimensional experiments (COSY, TOCSY, ROESY, HSQC and HMBC)

Residue	$\delta_{H-1/C-1}$	$\delta_{H-2/C-2}$	$\delta_{H-3/C-3}$	$\delta_{H-4/C-4}$	$\delta_{H-5/C-5}$	$\delta_{H-6/C-6}$
3-α-Rha*p* **A′**	5.11/103.0	4.27/68.4	4.08/78.2	3.59/71.4	3.89/69.7	1.33/17.5
3-α-Rha*p* **B′**	5.05/103.0	4.18/70.7	3.94/79.2	3.58/72.1	3.94/70.1	1.33/17.5
3-α-Rha*p* **C′**	5.04/103.0	4.18/70.7	3.94/79.2	3.58/72.1	3.94/70.1	1.33/17.5
3-β-Rha*p* **D′**	4.83/97.9	4.16/71.7	3.68/81.2	3.52/72.1	3.46/72.9	1.34/17.5

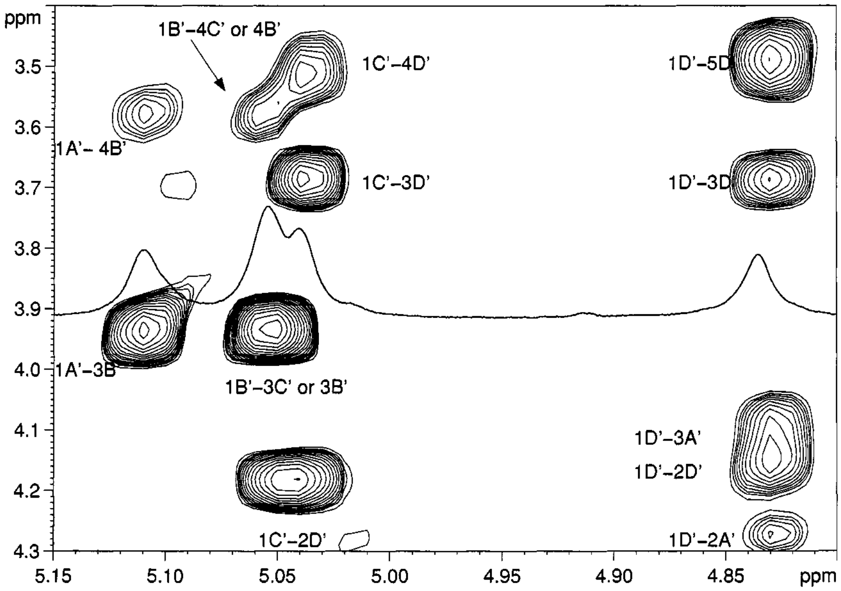
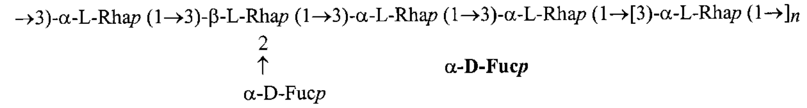


Figure 4. Part of the ROESY spectrum of the *Xanthomonas fragariae* SP (400 MHz, D_2O, 30 °C); all the inter- and intra-residual cross-peaks of the anomeric protons are shown



$$\begin{array}{l}\rightarrow 3)\text{-}\alpha\text{-L-Rha}p\ (1\rightarrow 3)\text{-}\beta\text{-L-Rha}p\ (1\rightarrow 3)\text{-}\alpha\text{-L-Rha}p\ (1\rightarrow 3)\text{-}\alpha\text{-L-Rha}p\ (1\rightarrow [3)\text{-}\alpha\text{-L-Rha}p\ (1\rightarrow]_n \\ \qquad\qquad\qquad\qquad\quad 2 \\ \qquad\qquad\qquad\qquad\quad \uparrow \qquad\qquad\qquad\quad \boldsymbol{\alpha\text{-D-Fuc}p} \\ \qquad\qquad\qquad\qquad \alpha\text{-D-Fuc}p \end{array}$$

B′(1→]$_n$ and [→3C′(1→3)D′(1→3)A′(1→3)B′(1→3)-B′(1→]$_n$.

Considering strictly the value of 3.5, these sequences should be alternated. However, since this is an average value it is not possible to rule out some degree of irregularity. In any case, the clear appearance of the NMR spectra (Figure 3) suggests a regular alternate structure for the major part of the backbone.

The above sequences were also in agreement with intraresidue dipolar couplings measured by ROESY experiments (Figure 4), which also supported the β-anomeric configuration of the D′ unit through the intraresidue NOEs measured among the H-1, H-3 and H-5 protons.

As far the OPS repeating unit was concerned, we showed that of the two types of terminal-D-Fuc*p* units, one is linked to position 2 of the β-Rha*p* residue and the other is located at the 2 position of an α-Rha*p* unit. The relative distribution of nodal β and α-Rha*p* residues was not determined and appears to be nonregular from the complex appearance of the NMR spectra (Figure 1 A and B), thatis, the location of fucose on α-Rha*p* residues is apparently random.

Conclusion

In conclusion on the basis of the chemical and spectroscopic data the above repeating unit for the most part of the *Xanthomonas fragaria* OPS could be suggested, where $n = 0$ or 1 alternatively and the other fucose unit (in bold) is located on one of the α-Rha residues of the repeating unit, in a casual position.

The nonregularity of the repeating unit of LPS which has been reported for some *Pseudomonas*[8] appears to occur in *Xanthomonas* as well. Actually this is, as we well know, the second example, the first being that of *Xanthomonas campestris*[4] already reported by us.

Experimental Section

General Methods: ^{1}H and ^{13}C NMR spectra were obtained in D_2O at 400 and 100 MHz, respectively, with a Bruker DRX 400 spectrometer equipped with a reverse probe, in the FT mode at 30 °C. ^{13}C and ^{1}H chemical shifts are expressed in δ relative to internal 1,4-

dioxane (δ = 67.4) and TSP (sodium 3-trimethylsilylpropionate-2,2,3,3-d_4), respectively. Two-dimensional spectra (COSY, TOCSY, ROESY, HSQC and HMBC) were measured using standard Bruker software. A mixing time of 200 ms was used in the ROESY experiment. GLC and GLC-MS separations were obtained with a Hewlett-Packard 5890 instrument fitted with a SPB-5 capillary column (0.25 mm × 30 m, Supelco), temperature program: 150 °C for 5 min., then 5 °C min^{-1} to 300 °C.

Growth of Bacteria, Isolation of LPS and OPS: Type strain NCPPB1469 of *Xanthomonas fragariae* was kept for long-term storage at −70 °C in 30% glycerol, and routinely grown on Wilbrink's medium slants.[9] Bacterial cell for the LPS extraction were obtained as previously reported.[4] The LPS fraction was obtained in the water phase (127 mg) by phenol/water treatment of the dried cells (2 g),[10] its lipopolysaccharidic nature was verified by fatty acid content[11] and Kdo assay.[12] In order to obtain the *O*-polysaccharide chain, the LPS was degraded with aq. 1% AcOH for 2 h at 100 °C and centrifuged at 11,000 rpm at 4 °C (1 h). The supernatant thus obtained (OPS fraction) was purified by GPC on a Bio-Gel P-10 column (90 cm × 1.5 cm) using 0.05 M ammonium bicarbonate buffer as eluent and monitored with a Waters differential refractometer.

Sugar and Methylation Analysis: The sugars were analysed as alditol acetates according to the following procedure: each sample was hydrolysed at 120 °C for 1 h with 2 M trifluoroacetic acid, evaporated under reduced pressure and reduced with $NaBH_4$. The alditol mixture was acetylated with acetic anhydride in pyridine. After workup, the sample was analysed by GLC-MS. The absolute configuration of the monosaccharides was determined according to the published method,[13] using GLC of acetylated (*S*)-2-octyl glycosides. Methylation analysis for the *O*-polysaccharide was carried out by standard procedures,[14] the hydrolysis of the methylated *O*-polysaccharide was performed with 2 M TFA (120 °C, 1 h) and the partially methylated monosaccharides, reduced with $NaBD_4$, were converted into alditol acetates with acetic anhydride in pyridine at 80 °C (30 min.) and analysed by GLC-MS.

Mild Hydrolysis: An aliquot of sample (10 mg) was submitted to mild hydrolysis which was performed in TFA (0.01 M) at 100 °C for 7 hours, then the sample was applied to a GPC column, Bio-Gel P2 (96 × 1.5 cm) eluted with water and monitored with a Waters differential refractometer. Two peaks were detected, the first (HP) eluted in the void volume (8 mg) and the second (2 mg) contained only fucose as reducing sugar.

Smith Degradation: The OPS fraction (40 mg) was treated with 0.05 M $NaIO_4$ (30 mL) at 4 °C for 96 h in the dark with stirring. The reaction was quenched with ethylene glycol and the crude mixture was reduced at room temperature overnight by addition of $NaBH_4$. The mixture was neutralised with AcOH, dialysed (cut-off 10 kDa) and lyophilised. The sample was then treated with 1% AcOH (20 mL) at 100 °C for 1.5 h. After evaporation the crude sample was purified on a Bio-Gel P-2 (96 × 1.5 cm) column, eluted with water and monitored with a Waters differential refractometer. The only peak (SP) detected (20 mg) was submitted to chemical and spectroscopical analysis.

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Received July 17, 2000
[O00364]