ELSEVIER

Carbohydrate Research 337 (2002) 1119–1127

CARBOHYDRATE RESEARCH

www.elsevier.com/locate/carres

# Structure of the phenol-soluble polysaccharide from *Shewanella putrefaciens* strain A6☆

Alexander S. Shashkov,[a,b] Vladimir I. Torgov,[a,b] Evgeny L. Nazarenko,[c] Vladimir A. Zubkov,[c] Natalya M. Gorshkova,[c] Raisa P. Gorshkova,[c] Göran Widmalm[a,*]

[a] *Arrhenius Laboratory, Department of Organic Chemistry, Stockholm University, S-106 91 Stockholm, Sweden*
[b] *N.D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Moscow 117913, Russian Federation*
[c] *Pacific Institute of Bioorganic Chemistry, Far East Branch of the Russian Academy of Sciences, Vladivostok 690022, Russian Federation*

Received 14 January 2002; accepted 10 April 2002

**Abstract**

The structure of the phenol-soluble polysaccharide from *Shewanella putrefaciens* strain A6 has been elucidated. Chemical modifications of the polymer in conjunction with $^{1}H$ and $^{13}C$ NMR spectroscopy, including 2D techniques, were employed in the analysis. It is concluded that the repeating unit is composed of two nine-carbon sugars as follows: →4)-α-Non*p*A-(2→3)-β-Sug*p*-(1→ where α-Non*p*A is 5-acetamido-7-acetamidino-8-*O*-acetyl-3,5,7,9-tetradeoxy-L-*glycero*-α-D-*galacto*-non-2-ulosonic acid (8eLeg) and β-Sug*p* is 2-acetamido-2,6-dideoxy-4-*C*-(3′-carboxamide-2′,2′-dihydroxypropyl)-β-D-galactopyranose, with the proposed name Shewanellose (She). © 2002 Elsevier Science Ltd. All rights reserved.

*Keywords:* *Shewanella putrefaciens*; Polysaccharide; NMR spectroscopy; Nonulosonic acid; Shewanellose; Biosynthesis

## 1. Introduction

*Shewanella putrefaciens* is an important Gram-negative bacterium which is found in many environments such as sediments,[1] oil-fields,[2] and foods.[3,4] It plays a major role in the turnover of nutrients in different ecological niches. The bacteria include, inter alia, marine and clinical isolates and organisms responsible for the spoilage of cold-stored, protein-rich foods. Their phenotypic and genotypic heterogeneity is well documented,[5,6] but their taxonomy remains unclear.[7] On the basis of DNA–DNA homology studies,[5] four groups of strains have been recognized: members of the two most clearly circumscribed groups (I and IV) can be differentiated by their DNA base composition and various phenotypic properties.

The extensive studies of bacterial polysaccharides have provided numerous examples of chemotaxonomic applications of compositional and structural data, but little work has been done on the *Shewanella spp*.[8,9] Some time ago, we determined the structure of a phosphorylated polysaccharide from *S. putrefaciens* strain S29.[10] In the present investigation, we report the results of the structural analysis of the unusual hydrophobic polysaccharide from *S. putrefaciens* strain A6.

## 2. Results and discussion

The wet bacterial cells were extracted by hot phenol–water. After centrifugation, the water and phenol phases were obtained and dialyzed separately. Sugar analysis of the water phase substance showed the presence of only mannose. Moreover, the $^{13}C$ NMR spectrum indicated that the water-soluble polymer represents a highly branched yeast-like mannan containing 2- and 6-linked D-mannose residues.[11] The phenol-phase soluble polymer was purified by dissolving it

☆ Presented in part at the Xth European Carbohydrate Symposium, Galway, Ireland, July 11–16, 1999 (abstract KN06).

* Corresponding author. Tel.: +46-8-163742; fax: +46-8-154908.
*E-mail address:* gw@organ.su.se (G. Widmalm).


PII: S0008-6215(02)00101-5

in a mixture of 2:1 methanol–chloroform, centrifugation to remove precipitates, evaporation of organic solvents, and lyophilization from water.

The $^{13}C$ NMR spectrum of the native phenol-soluble polysaccharide (PS) contained 26 signals. Three signals were observed in the region for anomeric carbons at $\delta_C$ 99.6, 104.4, and 105.8. Of these, the attached proton test[12] showed that only the carbon that resonates at 104.4 ppm carries a proton while the two other carbons are quaternary. From the same experiment, signals at 42.5 and 48.9 ppm could be shown to derive from methylene carbons and one more quaternary carbon was also identified at 79.4 ppm. The region for carbons bearing nitrogen contained three signals at $\delta_C$ 51.8 (2 C) and 57.8. Six resonances for methyl groups were observed upfield in the spectrum (15.5–23.8 ppm) and six signals from C=X groups, where X is O or N, were represented in the downfield region (168.0–177.9 ppm). A signal from a C=X group at 168.0 ppm, together with a signal from a methyl group at 20.3 ppm are characteristic of an *N*-acetamidinoyl group.[13] The chemical shifts of signals at 23.3 and 23.8 ppm are typical of *N*-acetyl groups and that at 21.8 ppm of an *O*-acetyl group.

Treatment of the native PS (**1**) with dilute aqueous ammonium hydroxide for 16 h led to *O*-deacetylation and to conversion of the *N*-acetamidinoyl group to an *N*-acetyl group.[14] As a result the $^{13}C$ NMR spectrum of the modified PS (**2**) contained one methyl and one C=X group less.

The $^1H$ NMR spectrum of the native PS showed, inter alia, signals for different methyl groups. One of them is typical of an *N*-acetamidinoyl group, $\delta_H$ 2.28, and three other signals located at about 2 ppm are characteristic of *N*-acetyl and *O*-acetyl groups. $^1H$ NMR spin systems of the native PS and the modified PS were assigned by $^1H,^1H$ COSY experiments and the results are given in Table 1. The $^1H$ and $^{13}C$ NMR chemical shifts and $^1H,^1H$ coupling constants indicated that one spin system originated from that of 8-*epi*-legionaminic acid (8eLeg)[15] with an *O*-acetyl group at C-8. Chemical shift data of the modified PS confirmed the position of *O*-acetylation and the conversion of the *N*-acetamidinoyl group into an *N*-acetyl group.

The remaining signals in the $^1H$ NMR spectra of the native and the modified PS could be assigned to three separate spin systems. The first spin system was of the AMX-type with chemical shifts in the native PS of 4.46, 4.13, and 3.88 ppm and coupling constants of $J_{AM}$ 7.3 and $J_{MX}$ 10.2 Hz. These coupling constants indicated a pyranosidic ring form with equatorial substituents. The second spin system was of the $A_3X$-type with chemical shifts of 1.18 and 3.80 ppm and a spin–spin coupling constant of 5.8 Hz. The third spin system was of the AB-type with chemical shifts of 2.24 and 2.33 ppm and a coupling constant of 15 Hz. $^1H,^1H$ NOESY spectra of the native (Fig. 1) and the modified PS indicated that the three isolated spin systems should belong to the

Table 1
Chemical shifts (ppm) in the $^1H$ NMR spectra of the polysaccharide from *S. putrefaciens* and modified products thereof

| Residue | Proton | **1** | **2** | **3** | **4** |
|---|---|---|---|---|---|
| 8eLeg | H-$3_{ax}$ | 1.74 | 1.76 | 1.70 (13.5) [a] | 1.76 (13) |
| | H-$3_{eq}$ | 2.76 | 2.37 | 2.53 (4.5) | 2.56 (4.5) |
| | H-4 | 3.55 | 3.59 | 3.51 (12.5) | 3.48 (12.5) |
| | H-5 | 3.67 | 3.57 | 3.54 (10) | 3.52 (9.5) |
| | H-6 | 4.62 | 4.49 | 4.40 (10) | 4.30 (10) |
| | H-7 | 3.73 | 3.70 | 3.77 (<2.5) | 3.75 (<2) |
| | H-8 | 5.14 | 3.88 | 5.08 (10) | 3.94 (8) |
| | H-9 | 1.35 | 1.21 | 1.35 (6) | 1.27 (6) |
| | Me [5] [b] | 2.00 | 1.96 | 1.98 | 2.00 |
| | Me [7] | 2.28 | 2.12 | 2.28 | 2.07 |
| | Me [8] | 2.02 | | 2.01 | |
| She | H-1 | 4.46 (7.3) | 4.44 | 4.96 (<2) | 5.00 (<2) |
| | H-2 | 4.13 (10.2) | 4.13 | 4.37 (<2) | 4.39 (<2) |
| | H-3 | 3.88 | 3.83 | 4.65 | 4.66 |
| | H-5 | 3.80 (5.8) | 3.79 | 4.03 (6.5) | 4.08 (6.5) |
| | H-6 | 1.18 | 1.18 | 1.26 | 1.31 |
| | H-4′a | 2.33 (15) | 2.32 | 2.52 (15.2) | 2.56 (15) |
| | H-4′b | 2.24 | 2.32 | 2.35 | 2.38 |
| | Me [2] | 2.01 | 2.03 | 1.96 | 1.98 |
| | MeO | | | 3.31 | 3.37 |

[a] $J_{H,H}$ values are given in Hz in parenthesis.
[b] Position of the carbon atom to which the acyl substituent having a methyl group is attached is given in square brackets.

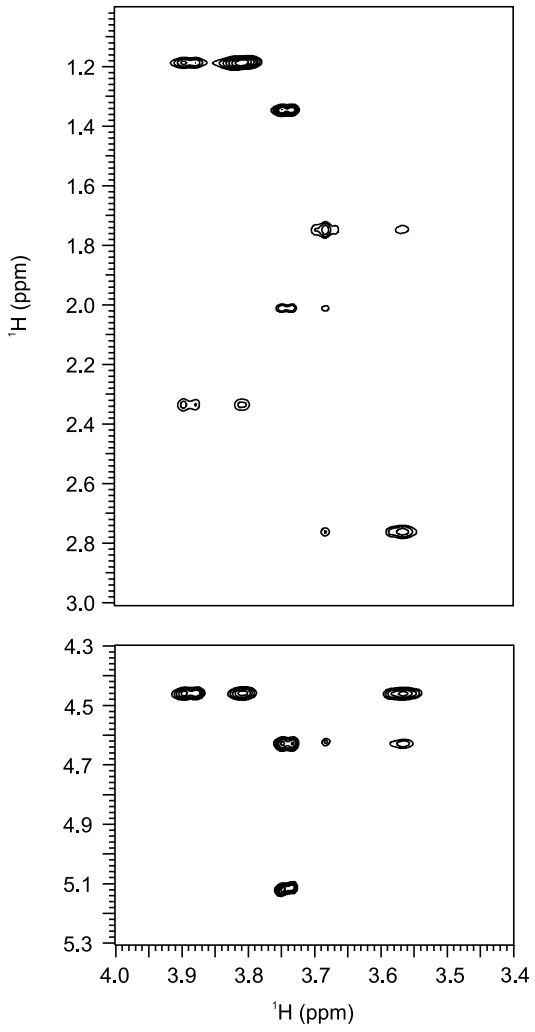



Fig. 1. Selected regions of the $^1$H,$^1$H NOESY NMR spectrum (mixing time 100 ms) at 45 °C of the native polysaccharide from *S. putrefaciens* strain A6.

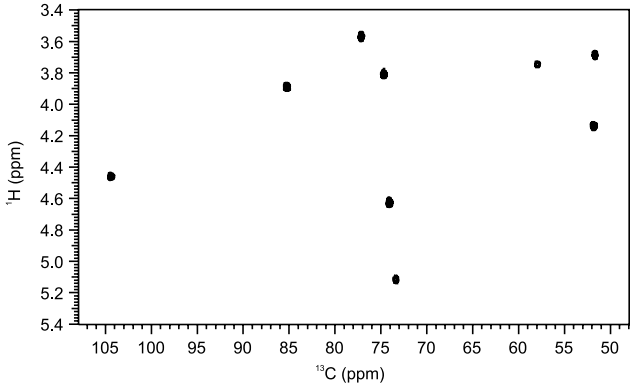



Fig. 2. Selected region of the $^1$H,$^{13}$C HSQC NMR spectrum at 45 °C of the native polysaccharide from *S. putrefaciens* strain A6.

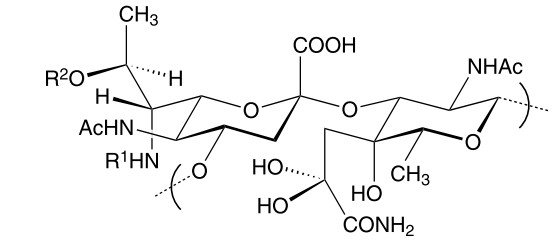
same novel sugar residue (She). The protons of the AB-system showed spatial proximity to protons in both the $A_3X$-system and the AMX-system. In particular, NOEs were observed between protons at $\delta_H$ 2.24/2.33 and 3.80/3.88 (four cross-peaks), but were absent between the former pair and $\delta_H$ 4.13. The proton at 4.46 ppm showed NOEs to the proton at 3.88 ppm in the same spin system and to the proton at 3.80 ppm of the second spin system, which indicate that these protons are axial and on the same side in a pyranosidic ring form. These observations allow us to suggest that the residue under consideration (Shewanellose, She) is a branched 6-deoxypyranose with an axial hydroxyl group and an equatorial three-carbon substituent at C-4, where the methylene group with the AB-spin system is bonded to C-4 of the pyranose.

$^1$H,$^{13}$C HSQC spectra of the native (Fig. 2) and the modified PS confirmed the presence of the 8-*epi*-legionaminic acid residue in the repeating unit of the polysaccharide. For example, the $^{13}$C chemical shifts of C-6 and C-8 in 8eLeg at $\delta_C$ 75.2 and 70.1, respectively,[15] are in close agreement to those observed in **2**. The downfield chemical shift of the C-4 signal at 77.0 ppm in the native PS and 78.3 ppm in the modified PS displayed the position of substitution in this residue, which was also in agreement with observed NOEs, i.e., a strong cross-peak between H-1 in She and H-4 in 8eLeg and a weaker one from H-1 in She to $H3_{eq}$ in 8eLeg. Furthermore, the signal at 104.4 ppm in the native PS and that at 104.3 ppm in the modified PS could be assigned to C-1 of She. The downfield chemical shift of the C-3 signal (85.2 ppm) is in a pyranosidic ring form characteristic of a substitution at the hydroxyl group and the chemical shift of C-2 (51.8 ppm) is typical of a carbon atom bearing nitrogen. Thus, the additional data indicate that She is a 2-amino-2,6-dideoxypyranose branched at C-4 having an equatorial linkage at the anomeric position.

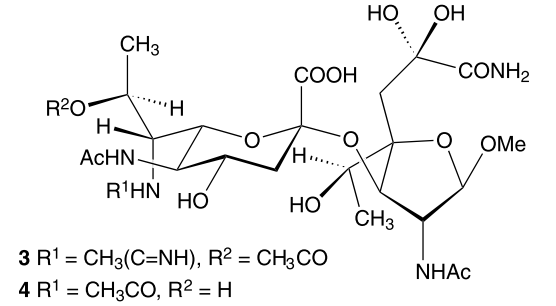
The $^1$H,$^{13}$C HMBC spectrum of the native PS was very informative in entangling the structure of the branched sugar. The spectrum showed three long-range correlations from the quaternary carbon (C-4) at 79.4 ppm to protons at $\delta_H$ 3.88 (H-3), 1.18 (H-6), and 2.24 (H-4′b). Heteronuclear couplings were also observed from H-4′b to C-3 and from H-4′a to C-5, supporting the branching at C-4 of She. Thus, the branching occurs at C-4, to which a methylene group is attached. The chemical shift of C-4 is typical for a carbon that has an oxygen bonded to it. This carbon should carry a hydroxyl group as an axial substituent since the $^{13}$C chemical shift of the nitrogen bearing C-2 in a sugar with the β-anomeric configuration is most similar to a residue with the *galacto*-configuration.[16] Taking into account the correlations observed in the HMBC spectrum from H-4′b to C-5′ ($\delta_C$ 105.8), and from H-4′a to C-6′ ($\delta_C$ 177.9), we propose a three-carbon chain as a substituent at C-4, namely, –$CH_2$–$C(OH)_2$–COX, where X is a non-carbon substituent.

Furthermore, the $^{1}H,^{13}C$ HMBC spectrum provided assignments of signals from non-proton bearing carbons as well as the location of acetyl and acetamidinoyl groups. In particular, the *N*-acetamidinoyl group, $\delta_H$ 2.28/$\delta_C$ 168.0, at C-7 of the 8eLeg residue, the position and orientation of C-1 of 8eLeg, via correlations between $\delta_H$ 1.74 and $\delta_C$ 176.5, as well as the confirmation of the an *O*-acetyl group, $\delta_H$ 2.02/$\delta_C$ 21.8, at C-8 of 8eLeg. Inter-residue correlations between H-1/C-1 of She and C-4/H-4 of 8eLeg, respectively, were in agreement with substitution of She at C-4 of 8eLeg (Fig. 3).

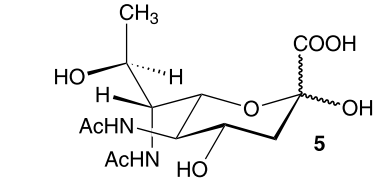
The assignment of downfield signals in the $^{13}C$ NMR spectrum of **1** allowed determination of carboxyl groups in the residues, using well-known dependence of their chemical shift on pH. The comparison of the spectra of the native PS at pD 7 and pD 1 (Table 2) showed that only one signal (C-1 of 8eLeg) significantly shifted in the region where carbonyl groups resonate. Consequently, the carbonyl group in the fragment branched at C-4 of She cannot be a carboxylic acid group.



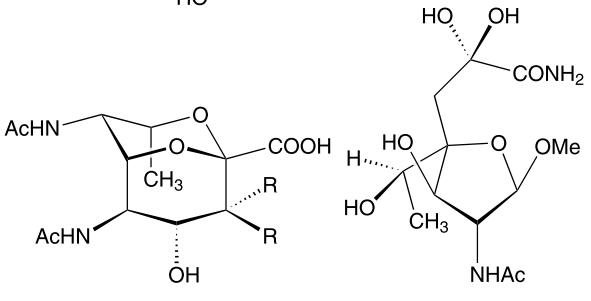

**1** $R^1 = CH_3(C{=}NH)$, $R^2 = CH_3CO$
**2** $R^1 = CH_3CO$, $R^2 = H$




**3** $R^1 = CH_3(C{=}NH)$, $R^2 = CH_3CO$
**4** $R^1 = CH_3CO$, $R^2 = H$




**5**




**6** R = H
**6-*d*$_2$** R = D

**7**

Fig. 3. Schematic of the native polysaccharide from *S. putrefaciens* strain A6 (**1**), synthetic 8-*epi*-legionaminic acid (**5**) and modified products thereof.

Methanolysis in anhydrous hydrogen fluoride of the native PS followed by gel-permeation chromatography (GPC) led to the isolation of a methyl glycoside of a disaccharide (**3**). Its $^{13}C$ NMR spectrum contained 27 signals (Table 2) and the $^{1}H$ NMR spectrum displayed, inter alia, an additional singlet at 3.31 ppm (3 H) in the region typical for an *O*-methyl group. The $^{1}H$ NMR spectrum was assigned using the same procedure as described for the native PS. The chemical shifts and coupling constants of signals from the 8eLeg residue were similar in the spectra from the disaccharide and the native PS. The resonances originating from the second residue (She) were quite different between the materials. Coupling constants $J_{H\text{-}1,H\text{-}2}$ and $J_{H\text{-}2,H\text{-}3}$ were $< 2$ Hz for the disaccharide and the $^{1}H$ chemical shifts of H-1 and H-3 differed significantly, $> 0.5$ ppm. In the $^{13}C$ NMR spectrum of the disaccharide, most resonances of the 8eLeg moiety were quite similar. However, a pronounced change was observed for C-4 from 77.0 ppm in the native PS to 67.3 ppm in the disaccharide revealing that 8eLeg is the terminal non-substituted residue. The signals of C-1–C-4 of She underwent significant downfield shifts in comparison to the native PS. Such changes are typical for a transformation from a pyranosidic to a furanosidic ring form, e.g., observed in the $^{13}C$ NMR spectra of methyl galactopyranoside to methyl galactofuranoside.[17]

The origin of the X-substituent in the COX group became clear after analysis by FABMS of the disaccharide which resulted in pseudomolecular ions $[M-H]^-$ at $m/z$ 660.2753 ($m/z$ calculated for $C_{27}H_{42}O_{14}N_5$ 660.2728) and $[M+H]^+$ at $m/z$ 662.352. Thus, a $CONH_2$ group is present, in agreement with the absence of pH-dependence on the $^{13}C$ chemical shift of the carbonyl carbon.[18] In addition, it is reasonable that the electron-withdrawing properties of this group favors hydration of the adjacent carbonyl group (cf. chloral hydrate). Also, a downfield resonance in the $^{13}C$ NMR spectrum for such a carbonyl group was not observed.

The above results are further corroborated by the presence of five $^{15}N$ resonances correlated to seven $^{1}H$ signals in a $^{1}H,^{15}N$ HSQC spectrum of **1** in 90% water/10% $D_2O$ (Fig. 4(A)). The assignments (Table 3) were supported by correlations in the $^{1}H,^{15}N$ HSQC-$^{1}H,^{1}H$ NOESY spectrum (Fig. 4(B)) and in the $^{1}H,^{13}C$ HSQC-$^{1}H,^{1}H$ NOESY spectrum (Fig. 5).

Saponification of the disaccharide under mild conditions gave a modified disaccharide (**4**). $^{1}H$ and $^{13}C$

Table 2
Chemical shifts (ppm) in the $^{13}C$ NMR spectra of the polysaccharide from *S. putrefaciens* and modified products thereof

| Residue | Carbon | **1** | **2** | **3** | **4** |
|---|---|---|---|---|---|
| 8eLeg | C-1 | 176.5 (175.7) [a] | 176.3 | 176.0 | 176.0 |
| | C-2 | 99.6 | 99.7 | 99.2 | 99.4 |
| | C-3 | 42.5 | 42.6 | 42.6 | 42.4 |
| | C-4 | 77.0 | 78.3 | 67.3 | 68.3 |
| | C-5 | 51.8 | 52.2 | 53.5 | 53.7 |
| | C-6 | 74.1 | 75.8 | 73.6 | 75.4 |
| | C-7 | 57.8 | 55.6 | 57.7 | 55.5 |
| | C-8 | 73.3 | 70.0 | 73.2 | 69.6 |
| | C-9 | 17.3 | 20.1 | 17.3 | 19.8 |
| | Me [5] [b] | 23.8 | 23.6 | 23.3 | 23.2 [d] |
| | C=O [5] [b] | 175.0 (175.0) | 174.4 [c] | 175.7 | 175.1 [e] |
| | Me [7] | 20.3 | 23.6 | 20.2 | 23.3 [d] |
| | C=X [7] | 168.0 (168.2) | 175.1 [c] | 167.8 | 175.2 [e] |
| | Me [8] | 21.8 | | 21.8 | |
| | C=O [8] | 174.6 (174.5) | | 174.4 | |
| She | C-1 | 104.4 | 104.3 | 111.2 | 111.2 |
| | C-2 | 51.8 | 51.8 | 60.9 | 60.7 |
| | C-3 | 85.2 | 85.2 | 92.0 | 91.8 |
| | C-4 | 79.4 | 79.3 | 99.5 | 99.5 |
| | C-5 | 75.7 | 74.5 | 71.0 | 70.7 |
| | C-6 | 15.5 | 15.3 | 18.1 | 18.0 |
| | C-4′ | 48.9 | 49.1 | 50.6 | 50.8 |
| | C-5′ | 105.8 (106.2) | 105.9 | 106.5 | 106.5 |
| | C-6′ | 177.9 (177.5) | 177.3 | 177.3 | 176.7 |
| | Me [2] | 23.3 | 23.9 | 23.3 | 23.2 |
| | C=O | 175.6 (175.4) | 175.3 | 174.2 | 174.5 |
| | MeO | | | 55.9 | 55.7 |

[a] Chemical shifts at pD 1 are given in parenthesis.
[b] Position of the carbon atom to which the acyl substituent is attached is given in square brackets.
[c] Pairwise interchangeable.
[d] Pairwise interchangeable.
[e] Pairwise interchangeable.

NMR data confirmed the transformation of the acetamidinoyl into an acetamido group and removal of the *O*-acetyl group. Comparison of the $^{13}C$ chemical shifts for the nonulosonic acid residue and synthetic 8eLeg (**5**) showed close agreement of signals for C-3–C-9 of the residue in the α configuration. This is in agreement with the above $^{1}H$,$^{13}C$ HMBC spectral data of the native PS.

Mild-acid hydrolysis of the modified disaccharide **4** in $D_2O$, monitored by $^{1}H$ NMR spectroscopy, resulted in a bis-deuterated monosaccharide with a spiroketal at C-2 with the proposed structure **6-*d*$_2$** and a methyl furanoside (**7**). The former showed in ESIMS a pseudomolecular ion $[M-H]^-$ at $m/z$ 317 originating from the 8eLeg residue. The latter revealed a pseudomolecular ion $[M-H]^-$ at $m/z$ 303 resulting from dehydration at C-5′, derived from the She residue. Synthetic 8eLeg **5** was subjected to treatment under acidic conditions at elevated temperature similar to the above described hydrolysis followed by HPLC to give **6**. Its tentative $^{1}H$ and $^{13}C$ NMR assignments are presented in Tables 4 and 5, respectively. The chemical shifts of **6-*d*$_2$** were identical, within experimental error, to those of **6** thereby confirming the product formed upon hydrolysis of **4**. The tentative $^{1}H$ and $^{13}C$ NMR chemical shifts of **7** (Tables 4 and 5) are similar to those in **4**, also for C-3 having $\delta_C$ 90.1 in **7** and $\delta_C$ 91.8 in **4**. However, it is well known that substitution via a ketosidic linkage induces only minor chemical shift changes at the aglyconic carbon.[19]

The absolute configuration of 8eLeg was inferred on the basis of its optical rotation value in different derivatives. Taking into account the optical rotation of disaccharide **4** ($[\alpha]_D$ +81°), and that the contribution of the furanose fragment is small ($[\alpha]_D$ +5° for **7**), we can conclude that the optical rotation of 8eLeg in **4** is large and positive. Furthermore, both **6** and **6-*d*$_2$** show positive optical rotations. Thus, in addition to the identity

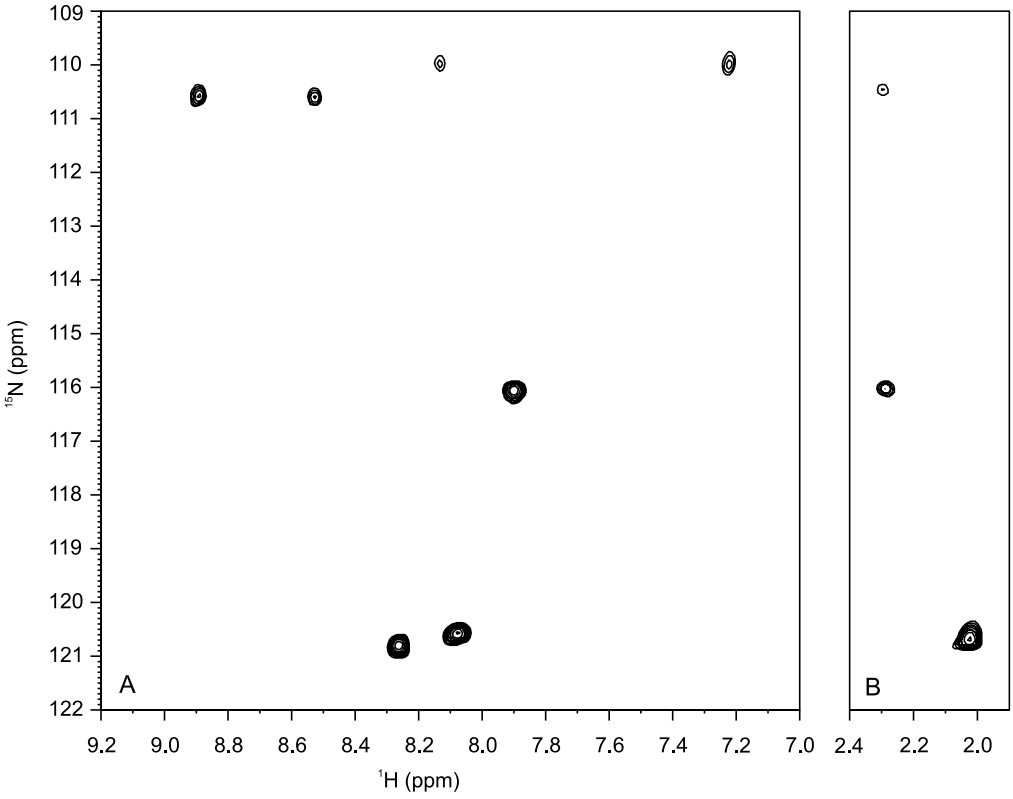



Fig. 4. (A) $^{1}$H,$^{15}$N HSQC spectrum of **1** in 90% water/10% $D_2O$ at 27 °C; (B) selected region of the $^{1}$H,$^{15}$N HSQC-$^{1}$H,$^{1}$H NOESY spectrum (mixing time 100 ms).

revealed by $^{1}$H and $^{13}$C NMR chemical shifts, their absolute configurations should be the same. Consequently, it is concluded that the 8eLeg residue has the L-*glycero*-D-*galacto*-configuration.

The absolute configuration of the novel sugar (She) could be determined using $^{1}$H,$^{1}$H NOESY data and glycosylation effects in $^{13}$C NMR spectra. In the native polysaccharide **1**, a strong NOE was observed from H-1 in She to H-4 together with a weaker one to H-3$_{eq}$ of 8eLeg indicates the D absolute configuration of She,[20] which is also supported by the absence of a γ-gauche effect for C-3 (vide infra).[21] The same conclusion may be drawn from analysis of $^{13}$C glycosylation shifts ($\Delta\delta$).[22] Comparison of the $^{13}$C chemical shifts in the modified polysaccharide **2** to those of 8eLeg as its sodium salt in the α-anomeric form[15] reveal for C-3, C-4, and C-5 of the nonulosonic acid $\Delta\delta$ of $+0.9$, $+8.6$, and $-2.0$, respectively. These $^{13}$C glycosylation shifts are characteristic of the D absolute configuration of She (cf. table 3 in Ref. 22). In addition, the $^{13}$C chemical shift of C-1, $\delta$ 104.4, indicates a significant displacement for this resonance upon glycosylation.

The repeating unit of the native PS from *S. putrefaciens* strain A6 is consequently: →4)-α-Non*p*A-(2→3)-β-Sug*p*-(1→ where α-Non*p*A is 5-acetamido-7-acetamidino-8-*O*-acetyl-3,5,7,9-tetradeoxy-L-*glycero*-α-D-*galacto*-non-2-ulosonic acid (8eLeg) and β-Sug*p* is 2-acetamido-2,6-dideoxy-4-*C*-(3′-carboxamide-2′,2′-dihydroxypropyl)-β-D-galactopyranose (She). This polysaccharide is unusual in several aspects. First, its physico-chemical properties renders it to be extracted into the phenol phase upon partition between phenol and water. Second, the repeating unit contains two nine-carbon sugars. Third, one of these is a novel *C*-branched sugar, an unusual structural peculiarity occurring, e.g., in sugars from the lipopolysaccharides of *Pseudomonas caryophylli*[23] and *Helicobacter pylori*.[24] Fourth, during biosynthesis sugar nucleotides of hexos-4-uloses are common intermediates in transformations into different monosaccharides.[25] Thus, the key biosynthetic step in formation of She can presumably take

Table 3
$^{15}$N and $^{1}$H chemical shifts (ppm) of the polysaccharide (**1**) from *S. putrefaciens*

| Residue | Nitrogen | $\delta_N$ | $\delta_H$ |
|---|---|---|---|
| 8eLeg | N-5 | 120.8 | 8.25 |
| | N-7 | 116.1 | 7.90 |
| | N=C [a] | 110.6 | 8.52, 8.88 |
| She | N-2 | 120.6 | 8.06 |
| | N-9 | 110.1 | 7.21, 8.12 |

[a] Acetamidinoyl nitrogen.

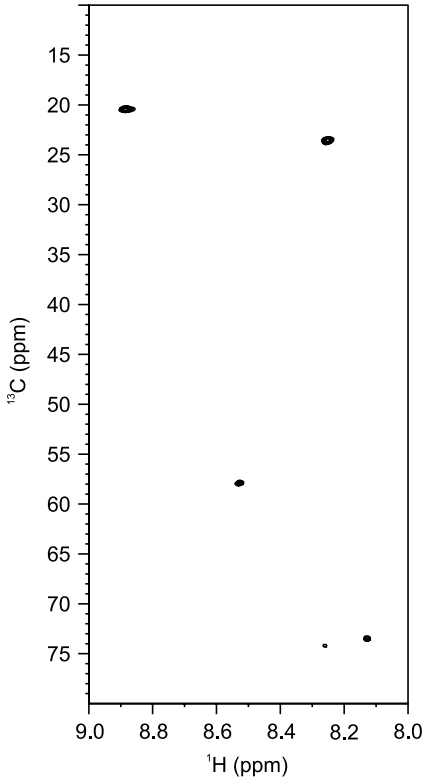



Fig. 5. Selected region of the $^{1}$H,$^{13}$C HSQC-$^{1}$H,$^{1}$H NOESY spectrum (mixing time 120 ms) of **1** in 90% water/10% $D_2O$ at 27 °C.

Table 4
Chemical shifts (ppm) in the $^{1}$H NMR spectra of modified products from synthetic 8eLeg and the polysaccharide of *S. putrefaciens*

| Proton | **6** | **7** |
|---|---|---|
| H-1 | | 5.04 |
| H-2 | | 4.35 |
| H-3 | | 3.50 |
| H-3a | 2.87 (16.9, 8.6) [a] | |
| H-3b | 2.97 (2.4) | |
| H-4 | 4.17 | |
| H-4′a | | 4.16 (15) |
| H-4′b | | 2.38 |
| H-5 | 3.92 (3) | 3.97 |
| H-6 | 3.85 (3) | 1.29 |
| H-7 | 3.86 (2) | |
| H-8 | 4.14 (6.5) | |
| H-9 | 1.09 | |
| Me [2] [b] | | 1.99 |
| Me [5] | 2.00 [c] | |
| Me [7] | 2.07 [c] | |
| MeO | | 3.38 |

[a] $J_{H,H}$ values are given in Hz in parenthesis.
[b] Position of the carbon atom to which the acyl substituent having a methyl group is attached is given in square brackets.
[c] Interchangeable.

place formally via a reaction between phosphoenolpyruvate and 2-acetamido-2,6-dideoxy-D-*xylo*-hexos-4-ulose. Notably, the latter ulose was even found to be present, with the same absolute configuration as She, in the capsular polysaccharide of *Streptococcus pneumoniae* type 5.[26]

## 3. Experimental

*General.*—NMR spectra were recorded in $D_2O$ at 30 °C (unless otherwise indicated) using JEOL GSX-270, Bruker DRX 500 and Varian Inova 600 MHz ($^{1}$H frequency) instruments. Chemical shifts are reported in ppm relative to acetone $\delta_H$ 2.225 and $\delta_C$ 31.45. Experiments were performed according to standard pulse sequences and data processing was carried out using software supplied by the manufacturer. The NMR experiments of **1** (20 mM, with respect to the molecular mass of the repeating unit) in 90% water/10% $D_2O$ were carried out on a Bruker 500 spectrometer equipped with a CryoProbe and the $^{1}$H and $^{15}$N resonances were referenced to internal sodium 3-trimethylsilyl-(2,2,3,3-$^{2}H_4$)-propanoate (TSP, $\delta_H$ 0.00) and external ammonia ($\delta_N$ 0.0),[27] respectively. Fast-atom bombardment mass spectrometry (FABMS) was performed on a JEOL SX-102 using a mixture of glycerol and thioglycerol in the positive mode and glycerol in the negative mode as a matrix and CsI as an internal standard. Electrospray-ionization mass spectrometry (ESIMS) was performed in the negative mode on a VG Quattro triple quadrupole mass spectrometer (Micromass).

*Bacterial growth and isolation of polysaccharides.*—The *S. putrefaciens* strain A6 was grown in Yoshimizu–Kimura medium.[28] The wet bacterial cells were conventionally extracted with 45% phenol and centrifuged. Water and phenol layers were dialyzed separately, concentrated, and freeze-dried. The phenol-phase material (1 g) was suspended in a mixture of MeOH and $CHCl_3$ (10 mL). The precipitate was removed by centrifugation and the supernatant solution was concentrated, redissolved in water, and freeze-dried (800 mg).

*HF-methanolysis.*—The native PS (52 mg) was subjected to methanolysis in anhyd hydrogen fluoride (2 mL MeOH and 8 mL HF) for 15 min at 22 °C, whereafter the solvent was removed under vacuum. The product was dissolved in water (15 mL) and freeze-dried. Subsequent purification of the material was performed by GPC on a Superdex 30 column with a pyridinium–acetate buffer. The major product was collected and freeze-dried to yield disaccharide **3** (22 mg), $[\alpha]_D$ + 50° (*c* 2.2, water).

*Disaccharide saponification.*—Disaccharide **3** (17 mg) was saponified in $D_2O$ (0.4 mL) at pH 12, obtained by addition of $Na_2CO_3$, at 20 °C for 14 days, purified by

HPLC on a Fractogel TSK-40W column to yield **4** (13 mg), $[\alpha]_D$ +81° (*c* 1.3, water).

*Disaccharide hydrolysis.*—Disaccharide **4** (13 mg) was dissolved in 0.5 M $CD_3COOD$ (0.4 mL) and kept at 100 °C for 6.5 h. The hydrolysis was monitored by $^1H$ NMR experiments. After lyophilization, the products were separated by HPLC on a column of Fractogel TSK-40W to give **6-*d*$_2$** (6 mg), $[\alpha]_D$ +39° (*c* 0.8, water) and **7** (4 mg), $[\alpha]_D$ +5° (*c* 0.4, water).

*Hydrolysis of 8-*epi*-legionaminic acid.*—Synthetic 8eLeg (20 mg) was dissolved in 0.5 M $CD_3COOD$ (0.7 mL) in a 90% water/10% $D_2O$ mixture and kept at 100 °C for 5.5 h in an NMR tube. Also, in this case the hydrolysis was monitored by NMR spectroscopy. The dark brown solution was evaporated under vacuo, co-evaporated with water twice to remove traces of AcOH and dissolved in water (20 mL). The sample was decolorized by a Sep-Pak C-18 column and evaporated under vacuo. Purification was performed by HPLC on a Supelco C-18 semi-preparative column in 0.05% $CF_3COOH$ to yield **6** (4 mg), $[\alpha]_D$ +17° (*c* 0.4, water).

## Acknowledgements

A.S.S. and V.I.T. thank Stockholm University for fellowships. This work was supported by a grant from the Swedish Research Council (VR). We thank Dr B.F. Vogel (Lyngby, Denmark) for the *S. putrefaciens* strain A6, Dr Y.E. Tsvetkov (Moscow, Russia) for a sample of synthetic 8-*epi*-legionaminic acid, Ms P. Seffers for FABMS (Stockholm, Sweden), Professor P.-E. Jansson for ESIMS (Huddinge, Sweden) and Dr R. Weisemann (Karlsruhe, Germany) for NMR experiments on the native polysaccharide in $H_2O$.

Table 5
Chemical shifts (ppm) in the $^{13}C$ NMR spectra of modified products from synthetic 8eLeg and the polysaccharide of *S. putrefaciens*

| Carbon | **6** | **7** |
|---|---|---|
| C-1 | 174.0 | 111.2 |
| C-2 | 100.8 | 61.1 |
| C-3 | 43.7 | 90.1 |
| C-4 | 72.2 | 99.8 |
| C-5 | 50.6 | 71.3 |
| C-6 | 69.0 | 18.1 |
| C-7 | 55.1 | |
| C-8 | 70.7 | |
| C-9 | 17.2 | |
| C-4′ | | 48.8 |
| C-5′ | | 105.0 |
| C-6′ | | 177.9 |
| Me [2] [a] | | 23.2 |
| C=O [2] | | 174.4 |
| Me [5] | 23.3 [b] | |
| C=O [5] | 175.5 [c] | |
| Me [7] | 23.4 [b] | |
| C=O [7] | 176.0 [c] | |
| MeO | | 56.2 |

[a] Position of the carbon atom to which the acyl substituent is attached is given in square brackets.
[b] Pairwise interchangeable.
[c] Pairwise interchangeable.

## References

1. Myers C. R.; Nealson K. H. *J. Bacteriol.* **1990**, *172*, 6232–6238.
2. Semple K. M.; Westlake D. W. S. *Can. J. Microbiol.* **1987**, *33*, 366–371.
3. Molin G.; Ternström A. *J. Gen. Microbiol.* **1982**, *128*, 1249–1264.
4. Stenström I.-M.; Molin G. *J. Appl. Bacteriol.* **1990**, *68*, 601–618.
5. Owen R. J.; Legros R. M.; Lapage S. P. *J. Gen. Microbiol.* **1978**, *104*, 127–138.
6. Gilardi G. L. In *Nonfermentative Gram-Negative Rods. Laboratory Identification and Clinical Aspects*; Gilardi G. L., Ed.; Marcel Dekker: New York, 1985; pp 17–84.
7. Baumann P.; Gauthier M. J.; Baumann L. In *Bergey's Manual of Systematic Bacteriology*; Krieg N. R.; Holt J. G., Eds.; Williams and Wilkins: Baltimore, 1984; Vol. 1, pp 343–352.
8. Moule A. L.; Wilkinson S. J. *J. Gen. Microbiol.* **1989**, *135*, 163–173.
9. Sledjeski D. D.; Weiner R. M. *Appl. Environ. Microbiol.* **1991**, *57*, 2094–2096.
10. Shashkov A. S.; Senchenkova S. N.; Nazarenko E. L.; Zubkov V. A.; Gorshkova N. M.; Knirel Y. A.; Gorshkova R. P. *Carbohydr. Res.* **1997**, *303*, 333–338.
11. Kogan G.; Pavliak V.; Masler L. *Carbohydr. Res.* **1988**, *172*, 243–253.
12. Patt S. L.; Shoolery J. N. *J. Magn. Reson.* **1982**, *46*, 535–539.
13. Knirel Y. A.; Vinogradov E. V.; Shashkov A. S.; Dmitriev B. A.; Kochetkov N. K.; Stanislavsky E. S.; Mashilova G. M. *Eur. J. Biochem.* **1987**, *163*, 627–637.
14. Paramonov N. A.; Knirel Y. A.; Kochetkov N. K. *Bioorg. Khim.* **1991**, *17*, 1111–1115.
15. Tsvetkov Y. E.; Shashkov A. S.; Knirel Y. A.; Zähringer U. *Carbohydr. Res.* **2001**, *335*, 221–243.
16. Jansson P.-E.; Kenne L.; Widmalm G. *Carbohydr. Res.* **1989**, *188*, 169–191.
17. Bock K.; Pedersen C. *Adv. Carbohydr. Chem. Biochem.* **1983**, *41*, 27–66.
18. Rundlöf T.; Weintraub A.; Widmalm G. *Eur. J. Biochem.* **1998**, *258*, 139–143.
19. Bock K.; Vinogradov E. V.; Holst O.; Brade H. *Eur. J. Biochem.* **1994**, *225*, 1029–1039.
20. Lipkind G. M.; Shashkov A. S.; Mamyan S. S.; Kochetkov N. K. *Bioorg. Khim.* **1988**, *14*, 522–531.
21. Jansson P.-E.; Kjellberg A.; Rundlöf T.; Widmalm G. *J. Chem. Soc., Perkin Trans. 2* **1996**, 33–37.
22. Shashkov A. S.; Lipkind G. M.; Knirel Y. A.; Kochetkov N. K. *Magn. Reson. Chem.* **1988**, *26*, 735–747.

23. Adinolfi M.; Corsaro M. M.; De Castro C.; Lanzetta R.; Parrilli M.; Evidente A.; Lavermicocca P. *Carbohydr. Res.* **1995**, *267*, 307–311.
24. Kocharova N. A.; Knirel Y. A.; Widmalm G.; Jansson P.-E.; Moran A. P. *Biochemistry* **2000**, *39*, 4755–4760.
25. Shibaev V. N. *Adv. Carbohydr. Chem. Biochem.* **1986**, *44*, 277–339.
26. Jansson P.-E.; Lindberg B.; Lindquist U. *Carbohydr. Res.* **1985**, *140*, 101–110.
27. Wishart D. S.; Bigam C. G.; Yao J.; Abildgaard F.; Dyson H. J.; Oldfield E.; Markley J. L.; Sykes B. D. *J. Biomol. NMR* **1995**, *6*, 135–140.
28. Yoshimizu M.; Kimura T. *Fish Pathol.* **1976**, *10*, 243–259.