# Absolute Configuration of 8-Amino-3,8-dideoxyoct-2-ulosonic Acid, the Chemical Hallmark of Lipopolysaccharides of the Genus *Shewanella*[§]

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The novel monosaccharide 8-amino-3,8-dideoxyoct-2-ulosonic acid (Kdo8N) was isolated by methanolysis from the lipooligosaccharide of the marine Gram-negative bacterium *Shewanella pacifica*. After HPLC purification, the absolute configuration was determined by the Mosher ester method and proven to be 4*R*,5*R*,6*R*,7*R*. This established the D-*manno*-configuration of the monosaccharide.

Lipopolysaccharides (LPSs) are molecular hallmarks of Gram-negative bacteria, where they are present as main constituents of the outer cellular membrane. These vital and essential molecules play a fundamental role, since they are involved in all the interaction processes of the bacterial cell with the surrounding environment, as well as in colonization and pathogenicity events. The typical LPS architecture is organized into three distinct regions, termed lipid A, core, and O-chain, respectively, each characterized by its own structure, biosynthesis, and function.[1] The most conserved portion of the LPS is the lipid A, the glycolipid region that anchors the molecule to the membrane and that is the epitope recognized by the innate immune system machinery of the host organism. Higher structural variability is observed in the saccharidic moiety of the LPS, comprising an oligosaccharide portion and a polysaccharide, termed O-chain, which is the antigenic determinant and is recognized by the acquired immune system. Such chemical architecture is typical of the so-called "smooth-type LPSs". Nonetheless, in all wild-type and mutant Gram-negative strains, variable amounts of LPS molecules deficient of the O-chain are expressed, referred to as "rough-type LPSs" or, simpler, lipooligosaccharides (LOSs). The connection between the lipid A and the core oligosaccharide is held by a characteristic monosaccharide, 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo).[1] The latter, in turn, represents the chemical hallmark of LPSs; Kdo biosynthesis is a conserved process that is also very well studied and described.[2] Up to now, only a few cases are reported of LPSs in which the Kdo is not present as the first monosaccharide of the glycosidic portion. In particular, in *Acinetobacter hemolyticus* LPS, the Kdo residue is nonstoichiometrically replaced by its 3-hydroxy modification, the D-*glycero*-D-*talo*-octulosonic acid (Ko), while Kdo itself is present only as a branching residue of the oligosaccharide.[3] Remarkably, stoichiometric substitution of Kdo occurs only with a different residue, its 8-amino derivative (Kdo8N), in the core oligosaccharides of the LPSs from *Shewanella* species. Actually, it has been detected by Vinogradov et al. and by us in the LOS from the species *S. algae,*[4] *S. oneidensis*,[5] *S. pacifica*,[6] and *S. waksmanii* (unpublished data).

Bacteria belonging to the genus *Shewanella* are divided into more than 20 species, including both free-living and symbiotic forms, all isolated from various marine sources, such as water, sediments, fish, algae, marine animals and others. They are responsible for the spoilage of protein-rich foods, and two strains, *S. putrefaciens* and *S. algae*, are known as opportunistic pathogens of humans and marine animals and recognized as the causative agents of soft tissue bacteremia and sepsis. According to this information, the elucidation of the structure of *Shewanella* LPSs/LOSs is an important issue. Within this frame we carried out the structural characterization of the core oligosaccharide primary structure from *S. pacifica* KMM 3772 LOS. The oligosaccharide fraction was isolated by means of alkaline deacylation of the LOS and was characterized by means of chemical, NMR, and MS analyses.[6] In that case, as in the previous findings of Kdo8N, the absolute configuration of this monosaccharide was designated by NMR spectroscopy, exploiting its structural analogy with original Kdo. In the latter case the rule works on the basis of the presence of the L-*glycero*-D-*manno*-heptose (Hep) residue linked at *O*-5 of Kdo. In fact, an *inter*residual NOE correlation between the anomeric proton of Hep and *H*-7 of Kdo is possible only in the case of a D-configuration of the Kdo monosaccharide.[7]

Even though the previous approach can be intended as valid for Kdo and "in analogy" also for Kdo8N, in our opinion, the absolute configuration assignment of this novel and unique monosaccharide is a pivotal starting point in the studies toward the biosynthesis of such an important molecule as LPSs. Herein, the detailed stereochemical description of Kdo8N is furnished, working directly on the isolated monosaccharide and not only in analogy with the Kdo within the oligosaccharide.

The pure monosaccharide was isolated as its *O*-methyl glycoside derivative from the LOS of *S. pacifica*, which was initially dephosphorylated with 48% HF and then subjected to methanolysis. Subsequently, the sample underwent N-acetylation and eventual *O*-acetyl analogues were removed by O-deacetylation. The resulting *N*-acetyl- *O*-methyl glycosides were separated by reversed-phase HPLC (Supelco C-18, $H_2O$). The acidic conditions employed for recovering the Kdo8N **1** resulted in intramolecular esterification, and the monosaccharide was exclusively recovered as its bicyclo 1,5-lactone derivative **2** (1 mg, Scheme 1).

The purity of this product was proven by NMR spectroscopy. The high-resolution $^1$H NMR spectrum is presented in Figure 1. Compound **2** was fully characterized by means of complete one- and two-dimensional NMR analyses (see Supporting Information). The complete resonance pattern was established by means of a COSY spectrum, and, on the basis of this information, $\delta_C$ were obtained from a DEPT-edited $^1$H,$^{13}$C-HSQC and a $^1$H,$^{13}$C-HMBC spectrum. The NMR data for this compound are collected in Table 1.

The singlet at 3.18 ppm of a single anomeric *O*-methyl group and the concomitant absence of the resonance for a carboxym-

§ Dedicated to Prof. Matteo Adinolfi on the occasion of his 70th birthday.

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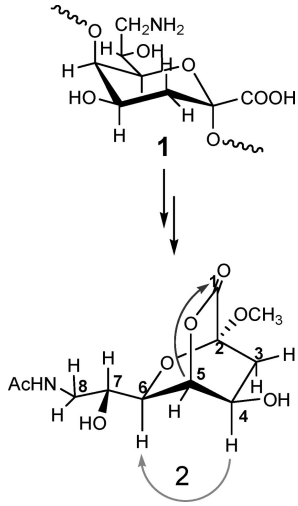
**Scheme 1.** Formation of the 1,5-Lactone **2** from Kdo8N **1** through Acid-Catalyzed Methanolysis, N-Acetylation, and O-Deacetylation (diagnostic NOE and long-range scalar correlations are indicated by arrows)




ethyl group, expected as a result of the hydrochloric acid treatment, pointed to the occurrence of a bicyclic form, which could arise by intramolecular esterification between the carboxyl group and either hydroxy group at *C*-5 (1,5-lactone ring) or *C*-7 (1,7-lactone ring). Similar results had been previously obtained in the case of acidic degradation of Kdo -containing oligosaccharides, where the formation of the 1,5-lactone had already been observed.[8] The formation of the bicyclic 1,5-lactone (compound **2**, Scheme 1) was confirmed by comparison of the chemical shift data with reference values[8] and by the observation of the NOE effect between *H*-4 and *H*-6, detected in the 2D ROESY spectrum, and by the scalar long-range correlation observed between the carboxylic group at *C*-1 (173.8 ppm) and *H*-5. The relative configuration of the molecule was described on the basis of the $^3J_{H,H}$ coupling constants deduced by the high-resolution $^1$H NMR spectrum, recorded at low temperature (Table 1), and through the observation of the NOE correlations. In particular, *H*-4 and *H*-6 possessed a pseudoaxial orientation, whereas *H*-5 was located at one of the bridging positions of the bicyclic system. On the basis of the structure depicted, lactone **2** presented only two free hydroxy groups, i.e., at *C*-4 and *C*-7. The appropriate stereochemical series was determined by spectroscopic methods according to Mosher's directives for absolute configuration determination of chiral alcohols.[9] This method requires particular attention when applied to molecules containing multiple stereogenic centers and showing unusual conformational restraints, as monosaccharides in general and lactone **2** in particular. Nevertheless, the suitability of such methodology in the case of the configurational analysis of monosaccharides had previously been proven in our laboratory.[10] Therefore, compound **2** was divided into two aliquots and converted into the corresponding bis-(*R*)- (**3**) and bis-(*S*)-α-methoxy-α-trifluoromethylphenylacetate (MPTA) (**4**) ester derivatives,[11] which were both fully analyzed by NMR. Downfield displacement of the resonances of *H*-4 and *H*-7 due to acylation was observed in both **3** and **4** in comparison with the original compound **2** (see Supporting Information). The evaluation of the absolute configuration for these two positions was then realized independently, assuming that no influence was exerted by the reciprocal position of the two MPTA ester groups, due to their mutual remote localization on the bicyclic system. The shielding and deshielding effects exerted by the MPTA ester moieties were evident by the upfield/downfield displacement observed for the protons vicinal to the ester moieties. In particular, comparison between the chemical shifts of homologous positions in **3** and **4** could be made. Complete proton and carbon chemical shift assignment for the two diasteroisomers was achieved through 2D NMR analyses. On the basis of these results, it was possible to evaluate the parameter $\Delta\delta_{R\text{-}S}$, defined as the chemical shift difference between analogous protons in the bis-(*R*)- and bis-(*S*)-MPTA ester derivatives. In particular, the MPTA ester group at *O*-4 induced resonance displacement of *H*-3 and *H*-5, whereas the presence of the ester group at *O*-7 shifted the *H*-8 and *H*-6 resonances.

The observed chemical shift differences among the two diastereoisomers are due to the effects depicted in Figure 2. As reported in Table 2, $\Delta\delta_{R\text{-}S} < 0$ were detected for *H*-3ax and *H*-8, whereas *H*-3eq, *H*-5, and *H*-6 exhibited $\Delta\delta_{R\text{-}S} > 0$. The apparent ambiguity regarding the $\Delta\delta_{R\text{-}S}$ value for *H*-3$_{eq}$ is not surprising and is explicable on the basis of further expansion of the Mosher methodology for carbohydrates, i.e, applying the rules to molecules with several stereogenic centers. In particular, the Mosher method was applied to the analysis of the absolute configuration of the monosaccharide caryophyllose.[10]

According to the relative, instead of the absolute, shifts of the proton resonances, a comparison of the $\Delta\delta_{R\text{-}S}$ for *H*-3$_{eq}$ and for *H*-5, it is evident indeed that a more sensitive downfield shift occurs for the latter signal. Thus, on the basis of this information, it is possible to establish the *R* configuration at both *C*-4 and *C*-7.

The relative configuration of the carbons composing the bicyclic system is known from the analysis of the coupling constant values in lactone **2** and in the monosaccharide **1** moiety within the oligosaccharide. Given these data, it was possible to assess that the configurations at *C*-5 and C-6 were both *R*. According to the accepted monosaccharide nomenclature, referring to the relative configuration of the stereogenic centers, this sequence is compatible with a D-*manno* configuration of Kdo8N, that is, the same configuration of Kdo of all LPSs from Gram-negative bacteria.

The analysis presented provides the only strict experimental assignment of the D- *manno* configuration of Kdo8N, and only at this stage can the hypothesis based on NMR analyses of the Kdo8N within the overall oligosaccharide structure be validated.[4–6] At the same time, this work is a further confirmation of the reliability of the Mosher method for the stereochemical analysis of monosaccharides, molecules intrinsically characterized by the occurrence of numerous stereogenic centers and several stereochemical restraints.

## Experimental Section

**General Experimental Procedures.** Reversed-phase HPLC purification of lactone **2** was performed on an Agilent 1100 series system equipped with a Supelcosil LC-18 column (Supelco) eluted with water and monitored by UV at 220 nm. NMR spectra of the HPLC-purified fractions were recorded in MeOH-$d_4$ at 281 K on a Bruker DRX-400 spectrometer. Spectra of the Mosher's ester derivatives **3** and **4** were executed at 295 K in $CHCl_3$-*d* solution on a Bruker DRX-600 equipped with a cryogenic probe.

**Extraction and Purification of the LOS from *Shewanella pacifica* KMM 3772.** Bacterial strains were isolated from the water samples collected in October and November 2000 from a depth of 1 m and 9–13 m (salinity, 32‰, temperature, 13.6 °C) using a standard hydrological plastic bathometer in different locations of Chazhma Bay, Gulf of Peter the Great, Sea of Japan, Pacific Ocean. Samples were kept at 4 °C and processed within 4–8 h. Bacteria were grown on a liquid medium containing (g/L) glucose (1), peptone (5), yeast extract (2.5), $K_2HPO_4$ (0.2), $MgSO_4$ (0.05), sea $H_2O$ (750 mL), and distilled $H_2O$ (250 mL). Cells were collected by centrifugation, washed with water, and next dried with acetone, obtaining 5 g of dried cells. The cells were extracted three times with a mixture of aqueous 90% phenol/$CHCl_3$/petroleum ether (2:5:8 v/v/v).[11] After removal of organic solvents under vacuum, the LOS fraction was precipitated from phenol with $H_2O$, the precipitate was washed with aqueous 80% phenol and three times with cold acetone, and then lyophilized (72 mg, yield: 1.6% of

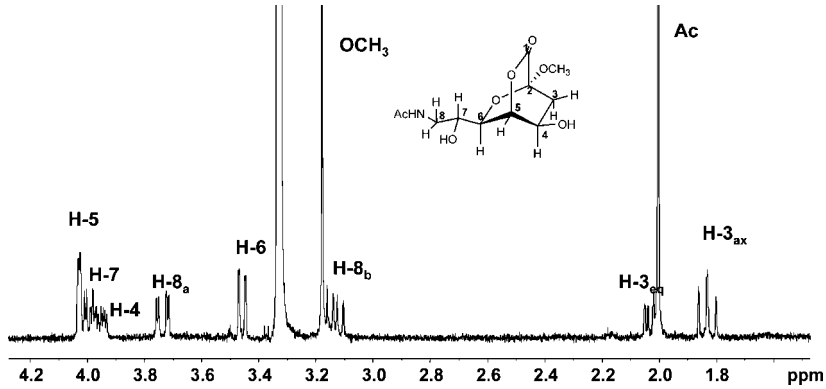



**Figure 1.** [1]H NMR spectrum of lactone **2** recorded at 400 MHz at 281 K in MeOH- $d_4$.

**Table 1.** [1]H and [13]C NMR Data (ppm, Hz) of Lactone **2**[a]

| proton | $\delta_H$ | $\delta_C$ |
|---|---|---|
| 1 | | 173.8 |
| 2 | | 99.8 |
| $3_{ax}$ | 1.83 t (12.0) | 34.0 |
| $3_{eq}$ | 2.03 dd (12.0, 5.0) | 34.0 |
| 4 | 3.96 m | 67.7 |
| 5 | 4.03 dd (2.4, 1.1) | 65.9 |
| 6 | 3.46 dd (1.1, 8.8) | 73.9 |
| 7 | 3.99 m | 67.1 |
| $8_a$ | 3.75 dd (3.5, 13) | 43.3 |
| $8_b$ | 3.13 dd (7.1, 13) | 43.3 |
| $OCH_3$ | 3.18 s | 50.9 |
| NHAc | 2.00 s | 22.2 (174.7)[b] |

[a] Spectra recorded in MeOH-$d_4$ at 400 ([1]H) and 100 ([13]C) MHz at 281 K. [b] Value in parentheses refers to C=O of the acetyl group.

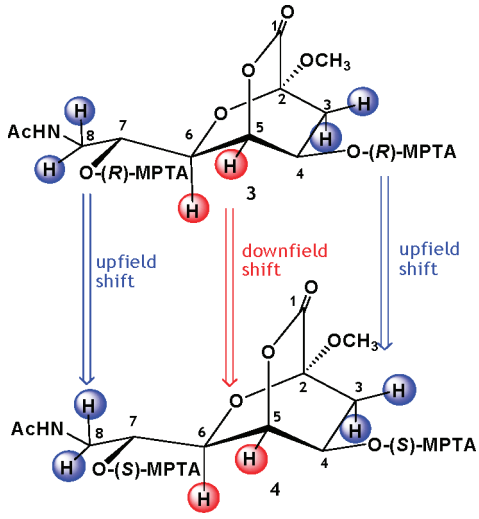



**Figure 2.** Comparison of the shielding/deshielding effects exerted by MPTA ester groups on the corresponding position of the 1,5-lactone of Kdo8N.

the bacterial dry mass). The purity of the sample was tested by silver-stained SDS-PAGE.

**Isolation of Kdo8N as Its 1,5-Lactone (2).** Eleven milligrams of pure LOS was subjected to dephosphorylation with aqueous 48% HF, under stirring at 25 °C for 3 h. After evaporation of the HF, the sample was washed with $H_2O$ (3×) and then subjected to methanolysis with 2 M HCl in MeOH, at 80 °C for 3 h. N-Acetylation was performed with $Ac_2O$, 50 μL in 500 μL of MeOH and 100 μL of pyridine, at 40 °C for 2 h. Eventual O-acetylated side products were removed by O-deacetylation with aqueous 33% $NH_4OH$ at 25 °C for 16 h. The *O*-methyl glycosides mixture was separated by RP HPLC. The fractions were characterized by means of 2D NMR spectroscopy. Spectra are presented in Figures S1–S3 in the Supporting Information.

**Table 2.** [1]H Chemical Shifts (ppm)[a] and $\Delta\delta_{R-S}$ for the Bis-(*R*)-(**3**) and Bis-(*S*)-MPTA (**4**) Ester Derivatives of Lactone **2**

| proton | bis-(*R*)-MPTA | bis-(*S*)-MPTA | $\Delta\delta_{R-S}$ |
|---|---|---|---|
| $3_{ax}$ | 2.07 | 2.17 | −0.10 |
| $3_{eq}$ | 2.35 | 2.26 | 0.09 |
| 4 | 5.42 | 5.25 | 0.17 |
| 5 | 4.49 | 4.04 | 0.45 |
| 6 | 3.53 | 3.11 | 0.42 |
| 7 | 4.36 | 4.42 | −0.06 |
| $8_a$ | 3.65 | 3.71 | −0.06 |
| $8_b$ | 2.65 | 2.78 | −0.13 |

[a] Spectra recorded in $CHCl_3$-*d* at 600 MHz on a Bruker DRX 600 equipped with a cryogenic probe.

**Preparation of the Mosher Ester Derivatives from Lactone 2.** The sample (1 mg) was divided into two aliquots, each solubilized in 100 μL of pyridine (ROMIL). The two samples were treated with 1.5 μL of enantiomerically pure (*S*)- and (*R*)-α-methoxy-α-trifluoromethylphenylacetic chloride at 25 °C. The reaction was followed by thin-layer chromatography (TLC) on Al plates precoated with Merck silica gel 60 $F_{254}$ as the adsorbent and quenched after 24 h by adding anhydrous MeOH. The bis-(*R*)- (**3**) and bis-(*S*)-MPTA-ester (**4**) derivatives were purified on Kieselgel 60 eluted with $CHCl_3$ to 1:1 $CHCl_3$/EtOAc. Compounds **3** and **4** were characterized by 2D NMR. [1]H NMR, [1]H,[13]C-HSQC, and COSY spectra for compound **3** and **4** are presented in the Supporting Information.

**NMR Spectroscopy.** ROESY spectra were measured using data sets (t1 × t2) of 4096 × 1024 points, and 16 scans were acquired. A mixing time of 200 ms was used. Gradient-selected COSY experiments were performed with 0.258 s acquisition time, using data sets of 4096 × 1024 points, and 64 scans were acquired. Total correlation spectroscopy experiments (TOCSY) were performed with a spin-lock time of 100 ms, using data sets (t1 × t2) of 4096 × 1024 points, and 16 scans were acquired. In all homonuclear experiments the data matrix was zero-filled in the F1 dimension to give a matrix of 4096 × 2048 points and was resolution enhanced in both dimensions by a shifted sine-bell function before Fourier transformation. HSQC and HMBC experiments were measured in the [1]H-detected mode via single quantum coherence with proton decoupling in the [13]C domain, using data sets of 2048 × 512 points, and 64 scans were acquired for each t1 value. Experiments were carried out in the phase-sensitive mode according to the described method. A 60 ms delay was used for the evolution of long-range connectivity in the HMBC experiment. In all heteronuclear experiments the data matrix was extended to 2048 × 1024 points using forward linear prediction extrapolation.

**Supporting Information Available:** Detailed spectroscopic characterization of compounds **2**–**4** is described. This material is available free of charge via the Internet at http://pubs.acs.org.

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