Characterization of a Novel Branched Tetrasaccharide of 3-Deoxy-β-manno-oct-2-ulopyranosonic Acid

THE STRUCTURE OF THE CARBOHYDRATE BACKBONE OF THE LIPOPOLYSACCHARIDE FROM ACINETOBACTER BAUMANNII STRAIN NCTC 10303 (ATCC 17904)†

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For the first time, the tetrasaccharide Kdo2(5Kdo2→5(5Kdo2→4)Kdo) (Kdo is 3-deoxy-β-manno-oct-2-ulopyranosonic acid) has been identified in a bacterial lipopolysaccharide (LPS), i.e., in the core region of LPS from Acinetobacter baumannii NCTC 10303. The LPS was analyzed using compositional analysis, mass spectrometry, and NMR spectroscopy. The disaccharide β-GlepNβ1→6β-GlepN, phosphorylated at O-1 and O-4', was identified as the carbohydrate backbone of the lipid A. The Kdo tetrasaccharide is attached to O-6' of this disaccharide and is further substituted by short L-rhamnoglycans of varying length and by the disaccharide β-GlepNAca1→4β-GlepNA (GlepNA, 2-amino-3-deoxy-2-glucopyranosuronic acid). The core region is not substituted by phosphate residues and represents a novel core type of bacterial LPS. The complete carbohydrate backbone of the LPS is shown in Structure I as follows:

Kdo2 → 4Kdo2 → 6GlepNa1 → 6GlepNa1 → P
5 4
↑ ↑
GlepNAca1 → 4GlepNAca1 → 4Kdo2
5
↑
1-Rhap+a1 → 3l-Rhap+a1 → 3l-Rhap+a1 → 3l-Rhap+a1 → 8Kdo2
Structure I

where Rha is rhamnose. Except were indicated, monosaccharides possess the d-configuration. Sugars marked with an asterisk are present in non-stoichiometric amounts.

Acinetobacter represents a coherent genus within the γ subclass of Proteobacteria in which it is placed within the family Moraxellaceae (1). Bacteria of this genus are widespread in Nature, where they occur in soil, water, food, and sewage as free-living saprophytic bacilli (2). However, they are also found in the hospital environment, e.g., on the skin of patients and staff (3), and some species have been recognized as dangerous nosocomial human pathogens that cause different diseases like pneumonia, meningitis, wound infections, septicemia, or endocarditis (4). Hospital strains appear to possess a significant capacity to survive on equipment and human skin for a long time. Moreover, in most cases they are multi-resistant against the major groups of antibiotics (2). On the basis of DNA-DNA hybridization, Acinetobacter is presently classified into 21 DNA groups (also called genomic species), 7 of which have been named to date, together with a number of un-grouped strains (5, 6). Of these 21 DNA groups, Acinetobacter baumannii is the main species associated with nosocomial infections, and most often resistant to antibiotics (2).

In common with other Gram-negative bacteria, Acinetobacter possess lipopolysaccharide (LPS) in the outer membrane of their cell wall; these LPSs represent chemotaxonomical and antigenic markers. In early investigations, it had been shown that this LPS is of the rough type, i.e., does not possess an O-specific polysaccharide (7, 8). Our further investigations of LPS from several hundred Acinetobacter strains using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver stain seem to confirm this; however, when these samples were Western blotted using polyclonal rabbit antisera against LPS, most samples gave a banding pattern characteristic for smooth LPS (9). In the meantime, we and others (Ref. 10 and

† The abbreviations used are: COSY, correlated spectroscopy; FAB-MS, fast atom bombardment-mass spectrometry; GlcNA, 2-amino-3-deoxyglucuronic acid; HPAEC, high performance anion exchange chromatography; Kdo, 3-deoxy-β-manno-oct-2-ulopyranosonic acid; LPS, lipopolysaccharide; NOE, nuclear Overhauser effect; TOCSY, total correlation spectroscopy; NOESY, nuclear Overhauser spectroscopy; HMQCS, heteronuclear multiple quantum coherence.
reference therein) have determined several structures of O-specific polysaccharides from various Acinetobacter genomic species, and we have further shown that rabbit antisera raised against a given LPS react highly specifically. It is still not fully understood why Acinetobacter S-form LPS cannot be stained using silver nitrate in SDS-polyacrylamide gel electrophoresis.

In general, the core region and lipid A represent a common structural unit occurring in all LPS; thus they are thought to be important for viability and membrane function of Gram-negative bacteria (11). Compared with the O-specific polysaccharide structures, the structural variability is more restricted within LPS from one genus. All core regions that have been investigated so far possess at least one residue of 3-deoxy-a-manno-oct-2-ulopyranosonic acid (Kdo) which links the core region to the lipid A. This Kdo residue may be substituted at O-4 by a second a-linked Kdo or an a-2→4-linked Kdo disaccharide (e.g. in LPS from Salmonella or Escherichia coli), and, in most cases that have been analyzed, it is elongated at O-5 with the rest of the core region, either via a manno-configured heptose (or in heptose-deficient cores) a hexose residue. Unique structures were identified in the core region of chlamydial LPS where either the trisaccharide Kdo2→8Kdo2→4Kdo (in Chlamydia trachomatis) or the tetrasaccharide Kdo2→8(Kdo2→4)-Kdo2→4Kdo (in Chlamydia psittaci) is present and no substitution at O-5 of any of the Kdo residues occurs (12). The branched Kdo tetrasaccharide has to date been the largest Kdo oligosaccharide identified.

The biosynthesis of lipid A and the inner core region has been investigated in detail in E. coli, and it has been found that the first two Kdo residues are transferred by a single Kdo transferase which is thus bifunctional (13). Similar results were obtained for the chlamydial Kdo transferases which transfer either three (in C. trachomatis and Chlamydia pneumoniae) or four (in C. psittaci) Kdo residues (12). Although these Kdo transferases are multifunctional enzymes, their primary structures have little similarity. In a recent investigation, the Kdo transferase genes from different strains of A. baumannii and Acinetobacter haemolyticus have been characterized (14). In in vitro assays it was shown that each of these enzymes transfers up to three Kdo residues to a synthetic lipid A precursor from E. coli, establishing their trifunctionality.

We have investigated the structure of the carbohydrate backbone of LPS from A. baumannii strain NCTC 10303 (ATCC 17904, formerly Acinetobacter calcoaceticus NCTC 10303), in which the presence of the trisaccharide 1-Rhap1→3-Rhap1→8Kdo had been reported in an earlier investigation (15). We now present the complete structure of this carbohydrate backbone, establishing a novel LPS core region comprising a novel branched Kdo tetrasaccharide that will help to extend our knowledge of multifunctional Kdo transferases.

EXPERIMENTAL PROCEDURES

**Bacteria and Bacterial LPS—** A. baumannii strain NCTC 10303 was cultivated as described (8). The LPS was obtained (yield: 3% of dry mass) from the lyophilized culture supernatant by a modified phenol/chloroform/light petroleum extraction as described (8).

**Preparation of Oligosaccharides—** The LPS (80 mg) was hydrolyzed in 5% acetic acid (100 °C, 5 h), and the precipitate was removed by ultracentrifugation (100,000 × g, 4 h). The supernatant was lyophilized (30 mg, 37.5% of the LPS) and contained a mixture of oligosaccharides which was separated on a column of Dowex 50-X4 (H+). Two fractions were obtained as follows: a neutral fraction, eluting with water, and a basic fraction, eluting with 5% aqueous ammonia. The basic fraction contained oligosaccharides 1 and 2, which were separated by using high performance anion exchange chromatography (HPAEC). The neutral fraction was reduced with NaBH4 and separated using gel permeation chromatography on TSK-40 in water, which yielded as one fraction oligosaccharide 3 and a second, low molecular mass fraction that was not further investigated.

For complete deacylation (16), the LPS (350 mg) was dissolved in anhydrous hydrxidine (8 ml), stirred for 30 min at 37 °C, cooled, poured into ice-cold acetone (80 ml), and centrifuged (2,500 × g, 30 min). The precipitate was washed twice with ice-cold acetone, dried, then dissolved in water, and lyophilized (de-O-acylated LPS, 256 mg, 67.4% of the LPS). An aliquot of this product (150 mg) was dissolved in 2 ml KOH (2 M), flushed with nitrogen for 1 h, and hydrolyzed in a sealed tube (100 °C, 16 h). After cooling and bringing to pH 6–7 with 2 M HCl, the precipitate was removed by centrifugation (2,500 × g, 30 min.), and the supernatant was desalted using gel permeation chromatography on TSK-40 in water. From this fraction, oligosaccharides 4–6 were isolated by HPSEC (5, 5, and 5 mg; 0.86, 1.43, and 1.43% of the LPS, respectively).

In a third experiment, the LPS (80 mg) was dissolved in anhydrous hydrxidine (3 ml), kept at 60 °C for 120 h, and evaporated to dryness. Water was added to this sample, and the insoluble fatty acids were removed by centrifugation (2,500 × g, 30 min), the supernatant of which was injected onto a reversed-phase HPLC Delta-Pak C18 (Waters) column (2 × 30 cm) that was eluted using water and from which oligosaccharide 7 was isolated (8 mg, 10% of the LPS).

**General and Analytical Methods—** Deamination of oligosaccharide 1 was performed with NaNO2 in 50% AcOH (1 h, 20 °C). Paper chromatography was carried out using pyridine/butanol/acetic acid/water (1:1:1:1, by volume). The conditions for gel permeation chromatography on TSK HW-20 were as described (17), and deamination was performed using NaNO2 in 30% AcOH (1 h, 20 °C). The semi-preparative HPAEC was performed as described (19) with the modification that the semi-preparative HPSEC column was eluted using gradient programs of 1–60% (oligosaccharides 1–3) or 30–80% (oligosaccharides 4–6) 1 M sodium acetate in 0.1 M NaOH in 80 min.

**Mass Spectrometry—** Gas chromatography and gas chromatography mass spectrometry were performed as described (17). Positive ion fast atom bombardment (FAB)-mass spectra were obtained using MS1 of a JEOL JMS-SX/SX102A tandem mass spectrometer operated at + or −10 kV accelerating voltage. The FAB gun was operated at 6 kV accelerating voltage with an emission current of 10 mA and xenon as the bombarding gas. Spectra were scanned at a speed of 30 s for the full mass range specified by the accelerating voltage used and were recorded and averaged on a Hewlett-Packard HP9000 data system running JEOL COMPLEMENT software. Collision induced dissociation mass spectra were recorded using the same machine, with helium as the collision gas in the third field free region collision cell, at a pressure sufficient to reduce the parent ion to one-third of its original intensity. The oligosaccharide was dissolved in 50 µl of water, and 1-µl aliquots of sample solution were loaded into a matrix of mono-thioglycerol, to which 1 µl of trifluoroacetic acid was added for the analysis of the untreated oligosaccharide. Negative ion mode electrospray mass spectrometry was performed on a VG Platform II single quadrupole mass spectrometer. Five µl of the oligosaccharide solution made for the FAB mass spectrum were diluted by addition of 10 µl of water, and 10 µl of this solution was infused into a mobile phase of acetonitrile/water (50:50, v/v) and introduced into the electrospray source at a flow rate of 5 µl min−1. Spectra were scanned at a speed of 10 s for m/z 700–1700, with a cone voltage of 60 V and recorded and processed using the Mass Lynx software, version 2.0.

**NMR Spectroscopy—** Structural assignments were made from sample solutions in D2O at 27 or 37 °C. For compounds 1–6, 1H and 13C NMR spectra were recorded at 600.13 MHz for 1H and 150.90 MHz for 13C with a Bruker AMX 600 spectrometer, and chemical shifts were reported relative to internal acetone (2.225 ppm for 1H and 31.5 ppm for 13C). The 1H,13C COSY spectrum was recorded at 101.25 MHz on a Bruker DRX 250 instrument at ambient temperature. Correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), nuclear Overhauser spectroscopy (NOESY), and heteronuclear multiple quantum coherence (HMQC) spectra were assigned using the computer program PRONTO (20). For compound 7, spectra were recorded on a solution of 5 mg in D2O with a Varian INOVA 750 spectrometer at 750.04 MHz for 1H and at 188.6 MHz for 13C at 27 °C. The double quantum-filtered correlation spectroscopy (COSY) spectrum was performed using the Varian standard program TNDQCOSY (21, 22), with 0.34 s acquisition time and 4 K data points. F1 was zero-filled to give a matrix of 4 × 2K points and was resolution-enhanced in both dimensions by a shifted sine bell. NOESY was performed using the Varian standard program TNOESY (23) with a mixing time of 200 ms. TOCSY was performed using the Varian standard program TNOTOCS (24, 25), with a spin-lock time of 120 ms. The heteronuclear single quantum coherence (HSQC) spectroscopy
copy was performed using the pulse field gradient standard Varian program GH5Q (26), with a gradient strength of 4, 4, and 2 G/cm and gradient time of respectively 2, 2, and 0.5 ms, respectively.

Conformational Analysis—Conformational analysis was performed using the hard sphere exonemeric-based GEGOP program (27, 28) at 800 and 1200 K with Metropolis Monte Carlo simulations with up to 3 × 10^6 steps. The a- and β-α-Kdo coordinates were taken from a published x-ray structure (29). For a semi-quantitative comparison with the observed NOE proton-proton distances, <r^2> were monitored. Furthermore, minimization and molecular dynamics were carried out for some oligosaccharides fragments using the computer program DISCOVER with the consistent valence force field (30, 31).

RESULTS AND DISCUSSION

Isolation and Chemical Analysis of the LPS—Most of the LPS from A. baumannii NCTC 10303 was released from the bacteria into the culture supernatant, from which it could be isolated using a modification of the phenol/chloroform/petroleum ether method (8). The additional extraction of dried bacteria gave less than 0.1% of the total LPS yield. Monosaccharide analysis of the LPS and determination of the absolute configurations of monosaccharides revealed the presence of L-Rha, d-Kdo, and d-GlcN. The configuration of Kdo was determined by measuring the optical rotation of its methyl ester, α-methylketoside derivative, isolated after mild methanolation of the LPS. Further compositional analyses of the LPS identified phosphorus, dodecanoic acid, 2-hydroxydodecanoic acid, and 3-hydroxydodecanoic acid, the last of which was the only amide-bound fatty acid in the lipid A, as revealed by fatty acid analysis of de-O-acetylated LPS.

Structural Analysis of Oligosaccharides 1 and 2 and Mixture 3—In an earlier investigation (15), the trisaccharide L-Rhapα1-3β-Rhapα1-8Kdo has been obtained from one LPS of A. baumannii NCTC 10303 after mild acidiy hydrolysis, dialysis, and separation of the dialysate on Bio-Gel P2. However, a second fraction possessing a higher molecular mass had additionally been isolated which contained Rha, Kdo, and phosphate but had not been investigated further. Consequently, the first step of the current investigation comprised hydrolysis of the LPS with 5% acetic acid in order to characterize the complete spectrum of products. Separation of the hydrolysate using ion exchange chromatography gave a basic fraction that was separated further using HPAEC that yielded oligosaccharides 1 and 2 and a neutral fraction that contained oligosaccharide mixture 3. The structures of oligosaccharides 1 and 2 were established using NMR spectroscopy (Tables I and II). The 1H assignments are based on two-dimensional phase-sensitive COSY and TOCSY and that of 13C on HMQC experiments. Anomeric configurations are assigned on the basis of the chemical shifts observed, and J1,2 values were determined from the COSY experiment (Table I). Oligosaccharide 1 consists of one residue each of 2,7-anhydro-3-deoxy-L-manno-2-ulofuranosic acid (2,7-anh-Kdo), a hexosaminuronic acid (13C NMR data, C-6 at 174.1 ppm in oligosaccharide 1) and a hexosamine. 2,7-anh-Kdo represents an artifact originating from acid hydrolysis of Kdo (32). It is identified by its 1H and 13C NMR chemical shifts and J1,2, H coupling constants that are in full agreement with those published (32). The hexosamine possesses the gluco-configuration, as revealed by its coupling constants J2,3 and J3,4 (~10 Hz). The coupling constants of the hexosaminuronic acid could not be determined from this sample due to signal overlapping; however, this was possible with oligosaccharide 2 which was identified as a partial structure of oligosaccharide 1, namely the reducing hexosamine hexosaminuronic acid. Thus, the hexosaminuronic acid also possesses the gluco-configuration (J2,3, 10 Hz; J3,4, 9 Hz). In both amino sugars, the amino group is located at C-2 as follows from the chemical shifts for C-2 at 54–59 ppm. Both, oligosaccharides 1 and 2 possess one N-acetyl group. As revealed by the chemical shifts of H-2, the amino group of the glucosamine is acetylated (3.814 ppm in oligosaccharide 1 and 3.822 ppm in 2), whereas that of the glucosaminuronic acid is free (2.71 ppm in oligosaccharide 1, 2.74 (α) and 3.02 (β) ppm in 2). The absolute configuration of glucosaminuronic acid could be determined as D using conformational analysis (see below); thus, the amino sugars in oligosaccharides 1 and 2 are 2-acetamido-2-deoxy-β-glucopyranose (d-GlcNAc) and 2-amino-2-deoxy-β-glucosaminuronic acid (d-GlcNAc). The sequence of monosaccharides in oligosaccharides 1 and 2 was determined by NOE measurements that gave NOE contacts between G1 and F4, and between F1 and E4 and E5. 2,7-anh-Kdo cannot be substituted at O-5 and hence must be glycosylated at O-4. This agrees with the 13C NMR chemical shift data that indicate that GlcNAc is a terminal residue (G in Fig. 1), and that GlcNAc (F) and 2,7-anh-Kdo (E) are each substituted at O-4 (signals for C-4 of GlcNAc at 76.1–76.8 ppm and of 2,7-anh-Kdo at 74.6 ppm). Taken together, the data allow the structures of oligosaccharides 1 and 2 to be assigned as depicted in Fig. 1.

Since the Kdo residue E is substituted by GlcNAc, the amino group that stabilizes the glycosidic bond, it cannot be liberated under conventional hydrolysis or methanolation conditions. Consequently, its absolute configuration was determined by measuring the optical rotation of 2,7-anh-Kdo, obtained after deamination of oligosaccharide 1 and paper chromatography. The optical rotation of the product was αD +63° which is close to that of the reference compound prepared from authentic Kdo (αD +68°) and allowed assignment of the α-configuration.

The presence of both oligosaccharides 1 and 2 can be explained by the finding that mild acid hydrolysis of Kdo yields 5-(β-erythro-1,2,3-trihydroxypropyl)-2-furoic acid and 2,7-anh-Kdo (32). The formation of the first product eliminates the substituent at O-4, resulting in compound 2, whereas the formation of 2,7-anh-Kdo yields trisaccharide 1.

The neutral fraction obtained by ion exchange chromatography of the LPS hydrolysate contained a mixture of several oligosaccharides with similar compositions. It was reduced with NaBH4 to give mixture 3, the NMR spectra of which contained signals for terminal and 3-substituted Rha, Kdo, and various signals of lower intensity, originating from Kdo derivatives (data not shown). Monosaccharide analysis of mixture 3 identified Rha and polyols and anhydropolysaccharides of Kdo. Methylation analysis identified terminal and 3-substituted Rha in the molar ratio of ~1:2.5. The ratio Rha:Kdo could not be determined because of the presence of multiple forms of Kdo. However, it may be concluded that mixture 3 consists of several oligosaccharides (predominantly tri- and tetrasaccharides) with structures similar to that of the previously identified trisaccharide L-Rhapα1-3β-Rhapα1-8Kdo (15).
unsaturated derivative as the result of alkaline β-elimination (1H and 13C NMR data, signals for H-4 at 5.723 ppm, C-4 at 107.5 ppm, and C-5 at 146.5 ppm). This was supported on prolonged alkaline hydrolysis (24 h at 120°) which converted purified oligosaccharides 6 to 5. Thus, the harsh alkaline conditions used for de-N-acylation induce the rarely occurring cis-elimination at the 4-substituted GlcNA residue.

Both oligosaccharides contain three Kdo residues, of which residues C and D were identified on the basis of the NOE connectivities observed between protons C3 and D6 that allowed identification of their α2-3-linkage (34). Kdo residue E is substituted by GlcNA, as indicated by the NOE connectivities between protons F1 and E4 and E5. Determination of the anomeric configuration of residue E was not straightforward. When compared with the chemical shifts of β-methyl Kdo (H-3eq, 2.388 ppm) (29), the low-field position of the E3eq proton

| TABLE I | 'H NMR chemical shift data (in ppm) and J H,H coupling constants (in Hz) observed for oligosaccharides derived from A. baumannii strain NCTC 10303 lipopolysaccharide |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Residue | H-1 | H-2 | H-3eq | H-4 | H-5 | H-6a | H-7 | H-8a | H-8b |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| A, in oligosaccharide | 5 | 5.461 | 3.052 | 3.696 | 3.522 | 4.069 | 3.657 | 4.227 |
| | 3.5 | 9 | 9 | 9 | 9 | 9 | 9 | 9 |
| A, in oligosaccharide | 6 | 5.594 | 3.278 | 3.841 | 3.665 | 4.139 | 3.716 | 4.273 |
| | 3.5 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| A, in oligosaccharide | 7 | 5.646 | 3.348 | 3.910 | 3.629 | 4.050 | 3.819 | 4.097 |
| | 8.5 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| B, in oligosaccharide | 5 | 4.631 | 2.863 | 3.696 | 3.613 | 3.635 | 3.355 | 3.382 |
| | 8.5 | 9 | 9 | 9 | 9 | 9 | 9 | 9 |
| E, in oligosaccharide | 5 | 1.765 | 1.906 | 4.115 | 4.006 | 3.636 | 3.949 | 3.722 | 3.965 |
| | 6 | 1.765 | 1.906 | 4.115 | 4.006 | 3.636 | 3.949 | 3.722 | 3.965 |
| E, in oligosaccharide | 7 | 1.744 | 1.998 | 4.031 | 3.989 | 3.557 | 3.926 | 3.716 | 4.003 |
| | 8.5 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| F, in oligosaccharide | 1 | 4.873 | 2.712 | 3.681 | 3.681 | 3.681 | 3.681 | 3.681 | 3.681 |
| | 2α | 3.5 | 5.281 | 3.071 | 3.882 | 3.721 | 4.122 |
| | 2β | 3.5 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| G, in oligosaccharide | 1 | 5.361 | 3.814 | 3.651 | 3.402 | 3.663 | 3.731 | 1.981a |
| | 2α | 3.5 | 10.5 | 10 | 10 | 10 | 10 | 10 | 10 |
| | 2β | 3.5 | 10.5 | 10 | 10 | 10 | 10 | 10 | 10 |
| H, in oligosaccharide | 7 | 5.046 | 4.070 | 3.844 | 3.453 | 3.838 | 1.292 |
| I, in oligosaccharide | 7 | 5.032 | 4.159 | 3.911 | 3.560 | 3.887 | 1.300 |
| L, in oligosaccharide | 7 | 4.841 | 4.090 | 3.850 | 3.552 | 3.748 | 1.300 |
| 3-OH-C12:0, in oligosaccharide | 7 | 2.481 | 4.022 | 1.515 | 1.280(H-5-H-11) | 0.860(H-12) |

a J = 0.3 Hz.  
Chemical shift for CH₃ of the N-acetyl group.
The absolute configuration of GlcNAc was deduced from conformational analyses of oligosaccharides 1 and 5–7 in which strong NOE connectivities between protons F1 and E4 and E5 were identified. The conformations of the disaccharides GlcNAc1–4(2,7-anh)-Kdo7 and GlcNAc1–4Kdo7 were calculated for both D- and L- absolute configurations of the constituents. The NOE signals observed (between F1 and E4 and E5) are only consistent with the D-configuration of both sugars, since short distances between these protons were determined on conformational analysis. In the L-configuration disaccharide, the distance between protons F1 and E5 was much greater. Therefore, the NOE signals observed identify the D-configuration of GlcNAc.

In the 31P NMR spectra of oligosaccharides 5 and 6, two peaks of similar intensity at 2.6 and 4.3 ppm were identified which, on 31P-1H COSY, gave correlations with protons A1 and B4, respectively. Thus, O-1 of GlcN residue A and O-4 of GlcN B are substituted by monophosphate groups.

Oligosaccharide 5 was additionally analyzed using positive and negative ion mode FAB and ES mass spectrometry. Positive ion FAB mass spectrometric analysis yielded a spectrum containing a cluster of intense pseudomolecular ions, separated from each other by increments of 22 mass units, at m/z 1318, 1340, 1362, 1384, 1406, and 1428. The ion at m/z 1318

**Table II**

| Residue | C-1 | C-2 | C-3 | C-4 | C-5 | C-6 | C-7 | C-8 |
|---------|-----|-----|-----|-----|-----|-----|-----|-----|
| A, in oligosaccharide |     |     |     |     |     |     |     |     |
| 5       | 93.5 | 55.9 | 72.1 | 70.7 | 72.9 | 70.8 |     |     |
| 6       | 91.8 | 55.3 | 70.8 | 70.0 | 72.4 | 70.5 |     |     |
| 7       | 91.3 | 55.0 | 70.1 | 69.8 | 73.0 | 68.3 |     |     |
| B, in oligosaccharide |     |     |     |     |     |     |     |     |
| 5       | 102.2 | 56.7 | 74.3 | 74.1 | 74.9 | 63.6 |     |     |
| 6       | 101.6 | 56.0 | 73.4 | 73.8 | 74.7 | 63.0 |     |     |
| 7       | 101.4 | 55.4 | 74.5 | 74.6 | 74.6 | 63.6 |     |     |
| C, in oligosaccharide |     |     |     |     |     |     |     |     |
| 5       | 175.8 | 103.2 | 33.7 | 73.1 | 69.5 | 73.6 | 70.2 | 64.5 |
| 6       | 175.8 | 100.9 | 35.4 | 66.7 | 67.7 | 72.7 | 71.6 | 63.6 |
| 7       | 174.7 | 102.8 | 34.8 | 65.9 | 67.2 | 72.4 | 71.5 | 63.4 |
| D, in oligosaccharide |     |     |     |     |     |     |     |     |
| 5       | 176.8 | 105.7 | 41.9 | 74.6 | 83.5 | 62.7 | 76.0 | 61.7 |
| 6       | 177.0 | 101.1 | 35.4 | 74.0 | 65.4 | 72.7 | 71.8 | 63.6 |
| 7       | 176.8 | 103.2 | 34.8 | 65.6 | 67.4 | 72.4 | 71.8 | 63.6 |
| E, in oligosaccharide |     |     |     |     |     |     |     |     |
| 1       | 99.8 | 55.9 | 75.8 | 76.7 | 74.3 | 174.1 |     |     |
| 2a      | 91.3 | 55.8 | 72.8 | 76.8 | 72.8 | 72.8 |     |     |
| 2b      | 98.0 | 58.5 | 77.9 | 76.1 | 75.9 | 75.9 |     |     |
| 5       | 98.0 | 53.8 | 67.5 | 107.5 | 146.5 | 170.0 |     |     |
| 6       | 94.4 | 55.0 | 73.8 | 76.6 | 73.3 |     |     |     |
| 7       | 91.5 | 54.0 | 71.2 | 75.2 | 72.3 |     |     |     |
| F, in oligosaccharide |     |     |     |     |     |     |     |     |
| 1       | 97.8 | 54.5 | 71.6 | 70.5 | 72.5 | 60.9 | 175.3 | 22.8 |
| 2       | 97.6 | 54.4 | 71.6 | 70.5 | 72.8 | 60.9 | 175.3 | 22.8 |
| 6       | 97.2 | 55.0 | 71.8 | 70.0 | 72.0 | 60.5 |     |     |
| 7       | 95.1 | 54.6 | 69.9 | 69.9 | 72.9 | 60.4 |     |     |
| G, in oligosaccharide |     |     |     |     |     |     |     |     |
| 1       | 102.7 | 70.6 | 70.5 | 72.4 | 69.5 |     |     |     |
| 7       | 102.6 | 70.4 | 78.8 | 71.7 | 69.6 |     |     |     |
| H, in oligosaccharide |     |     |     |     |     |     |     |     |
| 1       | 100.8 | 70.2 | 79.1 | 71.1 | 69.2 |     |     |     |
| 7       | 44.1 | 69.1 | 36.3 | 21–33(H-5-H-11)13.9(H-12) |     |     |     |     |

<sup>a</sup> Signals may be interchanged.
<sup>b</sup> Tentative assignment.
<sup>c</sup> Chemical shift of CO- of the N-acetyl group.
<sup>d</sup> Chemical shift of CH3- of the N-acetyl group.
corresponds to $[M + H]^+$ for a hexasaccharide of composition HexNH$_2$(Kdo)$_3$(GlcNH$_2$)$_2$(PO$_3$)$_2$ that lacks the element of water, thus corresponding to oligosaccharide 5. The ions having increments of 22 mass units consistent valence force field result from sodium salt formation and correspond to species bearing from one to five sodium atoms. Negative ion FAB mass spectrometric analysis yielded analogous results, with $[M + 2H]^+$ ions at $m/z$ 1382, 1404, 1426, and 1448 for the same species bearing from three to six sodium atoms. The negative ion mode ES mass spectrometric experiment yielded ions at $m/z$ 1360, 1382, 1404, 1426, 1448, 1470, and 1492 corresponding to the same structure bearing from two to eight sodium atoms. The site of dehydration was confirmed after incubation of the hexasaccharide with ammonia solution, a treatment that opens lactones. The positive FAB mass spectrum obtained from the product of this reaction bears ions at $m/z$ 1318, 1340, 1356, 1362, and 1378 (Fig. 3) demonstrating that while the procedure efficiently displaces sodium from the hexasaccharide, it does not result in the addition of the 18 mass units that would be expected on opening a lactone. Collision-induced dissociation
TABLE III
Calculated interatomic distances (in Å) and interresidual NOE contacts observed in oligosaccharides 5–7

| Protons | Calculated distances at 1200K | Observed NOE contacts between | 800K | 800K | 800K | 800K |
|---------|-----------------------------|-------------------------------|------|------|------|------|
| B1–A6  | 5.6,7                         | m                             | 2.51| 2.45| 2.68|      |
| C3ax–D6| 5.6,7                         | s                             | 2.32| 2.57| 2.18|      |
| C3eq–D6| 5.6,7                         | s                             | 3.25| 3.09| 3.60|      |
| C3eq–D7| 5.6,7                         | m                             | 3.33| 3.15| 3.61|      |
| C3eq–D8R| 5.6,7                        | w                             | 2.76| 2.78| 2.67|      |
| C3eq–D8S| 5.6,7                       | m                             | 2.73| 2.70| 2.68|      |
| C3eq–E6| 5.6,7                         | w                             | 3.89| 3.75| 4.38|      |
| C5–E4| 5.6,7                         | w                             | 2.12| 1.99| 4.29|      |
| D3ax–C5| 5.6,7                         | w                             | 4.15| 4.16| 4.20|      |
| D3eq–C5| 5.6,7                         | w                             | 4.41| 4.12| 8.14|      |
| D3eq–E4| 5.6,7                         | w                             | 2.75| 2.60| 6.60|      |
| D3eq–E5| 5.6,7                         | s                             | 4.42| 4.13| 6.14|      |
| D3eq–E6| 5.6,7                         | s                             | 2.84| 2.64| 4.66|      |
| D4–E4| 7.0                           | m                             | 2.58| 2.37| 6.08|      |
| D4–E5| 7.0                           | m                             | 4.34| 4.52| 8.08|      |
| E3ax–C5| 5.6,7                         | w                             | 3.59| 4.29| 3.15|      |
| E3eq–C5| 5.6,7                         | w                             | 4.90| 5.62| 5.34|      |
| E3eq–C6| 5.6,7                         | w                             | 6.01| 6.22| 5.71|      |
| E3eq–C7| 5.6,7                         | w                             | 4.21| 4.65| 3.81|      |
| E3eq–J6| 7.0                           | s                             | 2.89| 2.91| 2.87|      |
| E3eq–J6| 7.0                           | w                             | 4.49| 4.47| 4.46|      |
| E3eq–J6| 6.0                           | s                             | 2.28| 2.16| 2.44|      |
| E3eq–J6| 6.0                           | w                             | 2.62| 2.71| 2.61|      |
| E3eq–J6| 6.0                           | m                             | 3.15| 3.10| 3.20|      |
| E3eq–J5| 7.0                           | w                             | 4.16| 4.19| 4.17|      |
| E3eq–J5| 7.0                           | w                             | 3.30| 3.41| 3.35|      |
| E3eq–J6| 7.0                           | w                             | 5.73| 5.76| 5.76|      |
| E3eq–J6| 6.0                           | s                             | 4.40| 4.46| 4.44|      |
| E3eq–J6| 7.0                           | s                             | 3.77| 3.79| 3.85|      |
| E3eq–J7| 7.0                           | s                             | 2.17| 2.20| 2.25|      |
| F1–D3ax,eq| 7.0                       | w                             | 2.59| 2.37| 8.44|      |
| F1–D4| 5.6                           | m                             | 2.59| 2.37| 8.44|      |
| F1–E4| 5.6,7                         | s                             | 2.39| 2.44| 2.37|      |
| F1–E5| 5.6,7                         | s                             | 3.16| 3.05| 3.22|      |
| G1–F3| 6.7                           | m                             |      |      |      |      |
| G1–F4| 6.7                           | s                             |      |      |      |      |
| G1–F5| 6.7                           | w                             |      |      |      |      |
| H1–E3| 7.0                           | s                             |      |      |      |      |
| H1–L3| 7.0                           | s                             |      |      |      |      |
| I1–L3| 7.0                           | s                             |      |      |      |      |
| I1–J5a,8b| 7.0                       | s                             | 2.45| 2.46| 2.45|      |
| L1–J3R| 7.0                           | s                             | 2.65| 2.72| 2.73|      |
| L1–J3S| 7.0                           | s                             |      |      |      |      |

**The Structure of Oligosaccharide 7**—In order to obtain rhamnose-containing oligosaccharides, LPS was deacetylated with anhydrous hydrazine. Separation of the products on a C18 reversed-phase column yielded completely deacetylated products, eluting with the solvent front, and oligosaccharide 7, containing one 3-hydroxydodecanoic acid residue. NMR spectroscopy of the deacetylated products indicated structures similar to that of oligosaccharide 7; however, it was not possible to isolate a pure compound due to decomposition. Oligosaccharide 7 also decomposes at neutral pH over several days, mostly due to cleavage of Kdo J, but it is stable at pH ~8 and could thus be analyzed. The 1H, 13C, and 31P NMR spectroscopic analysis of oligosaccharide 7 showed that it contains the partial structure A–G, one additional Kdo J, and three rhamnose residues (H, I, J, and L). The structure of the fragment H-I-L-J is identical to that of oligosaccharide 3. To determine the attachment site of the 3-hydroxydodecanoic acid, a NOESY spectrum of a solution of oligosaccharide 7 in H2O:2H2O (9:1, by volume) was recorded. A strong NOE signal between the amide proton and H-2 of GlcN B was observed, indicating its substitution by the fatty acid. This acyl group is more stable to hydrazine deacetylation than that attached to GlcNA.

In the 13C NMR spectrum of oligosaccharide 7, a low-field shift of carbon E5 (69.7 ppm, compared with 63.5 ppm in oligosaccharide 6) indicated the attachment of J to O-5 of residue E. The NOESY spectrum indicated strong NOE connectivities between protons J3 and E7, and E3 and J6, in addition to those identified for oligosaccharides 5 and 6 (Fig. 4, Table III). In order to determine the structure and explain the NOE connectivities observed, a conformational analysis of the structural elements GlcNA1→4Kdo2→5(Kdo2→4)Kdo2→6GlcNA (F-E-D-C-B) and GlcNA1→4Kdo2→5(Kdo2→4)Kdo2→6GlcNA (F-E-D-C-B, GlcNA was set as residue instead of GlcNA) was performed using Metropolis Monte Carlo simulations and the program GEGOP (27, 28). The structure with residue J o2−7-linked to E showed observations with protons of these residues not in agreement with the calculations. The calculations reveal mainly a single minimum energy conformation for the glycosidic linkage of Kdo2→5-Kdoa (J-E) with ϕ ~–90° and ψ ~–20°, where ϕ represents the dihedral angle C1-C2-O2-C5 and ψ that of C2-O2-C5-H5 (Fig. 5). The calculated interatomic distances averaged as <r−6> are consistent with all NOE connectivities in which the protons of residue J take part. For the linkage Kdo2→5-Kdoa (E-D), two minima were observed. A calculation at 800K showed no transition between these two energy wells, and it is possible to obtain separate averaged distances for each conformation. One of these with ϕ and ψ near 20° resembles that of the J-E linkage and does not explain the experimental results (800K-2 in Table III), in particular the NOE connectivities observed between D3eq and E5, C5, and E6, and the absence of connectivities between E3 and C7, and C3 and E6. In contrast, the experimental data obtained are consistent with another conformation with ϕ ~–90°, ψ ~–20° for the E-C linkage (800K-1 in Table III). Calculations at higher temperature (1200 K) showed transitions between these two conformations and give average interatomic distances. Averaging as <r−6> emphasizes short distances, consistent with all NOE connectivities observed. However, averaging clearly predicted an NOE between E3 and C7, which is not observed. In conclusion, it is most likely that the linkage E-C exists in a conformation with ϕ approximately –90° and ψ approximately –20°, which is different from that of J-E.

Conformational analysis of the structural element GlcNA1→4Kdo2→5(Kdo2→4)Kdo2→6GlcNAβ (F-E-D-C-B) yielded a similar conformation, showing that the presence/absence of
residue J is of no significant conformational influence. The same two minima were obtained for the linkage E-C, of which only one gave interatomic distances consistent with the NMR data. The other interatomic distances are also consistent with the experimental observations. A similar conformation (Fig. 6) to that described above was obtained from another calculation using the consistent valence force field for the two structural elements possessing three (C, D, E) and four Kdo residues. Similarly, molecular dynamics simulations of the fragment 4Kdo2→5(Kdo2→4)Kdo (E-D-C) for up to 400 ps in a water box at 298 K starting at either minimum did not show any transitions between minima.

Fig. 3. Positive ion FAB-mass spectrum of oligosaccharide 5 after treatment with dilute ammonia solution.

Fig. 4. Part of the NOESY spectrum of oligosaccharide 7. The letters for the cross-peaks refer to the monosaccharide residues shown in Fig. 1, and the arabic numerals refer to the proton in the respective residue.
Unusual chemical shifts were observed for a number of protons of those Kdo units that are attached to position O-5 of another Kdo. As the major effect, a strong deshielding (0.7–0.9 ppm) of H-6 is observed for Kdo residue E, originating from the close proximity to the O-4 of Kdo C (distance between H-6 of E and O-4 of C, 2.1 Å, and between H-4 of E and O-4 of C, 2.8 Å; compare Fig. 6). This effect is probably due to a rigid arrangement of the branched Kdo tetrasaccharide, and it can be compared with the general effect observed in α-D-pyranoside residues linked to an axial hydroxyl group that is in close proximity to an equatorial hydroxyl group, as is present for example in galabiose (35). Furthermore, the equatorial H-3 is shifted downfield, but less strongly, which is probably due to an interaction with the lone pair of the glycosidic oxygen possessing a fixed orientation. The slight differences between the chemical shifts of protons H-6 and H-3 of Kdo residues E and J can be explained by the different conformation of the α2→5 linkage, also reflected in the differences observed for the NOE connectivities (thus an analogue of the NOE connectivity between protons 3eq and 7 is not observed for the pair E-C).

In conclusion, we have successfully established the chemical structure of a novel core type of LPS from *Acinetobacter* (Fig. 1) which comprises the tetrasaccharide Kdo2→5Kdo2→5-(Kdo2→4)Kdo (J-E-D-C) that has been identified for the first time. However, a Kdo2→5Kdo2 disaccharide had been identified in the core region of *Campylobacter lari* strain PC 637 (36). The core region of LPS from *A. baumannii* NCTC 10303 includes two structural elements in which a Kdo residue is connected to O-5 of another Kdo residue. The chemical behavior of these 5-linked Kdo residues was found to be different. The linkage of Kdo J, which is substituted by short rhamnose oligosaccharides is extremely acid-labile and is also cleaved by hot alkali, whereas that of residue E, which is substituted by GlcNAc2→4GlcNAc possesses a similar stability to that of the 4- and 6-linked Kdo residues. Conformational analyses of these structural elements showed that the conformation of the glycosidic linkages is different in the two Kdo2→5Kdo2 disaccharides, which could be a reason for the difference in linkage stability observed.

Interestingly, the presence of a branched trisaccharide Kdo-(Kdo)Kdo had been proposed for the core regions of LPS from *Salmonella enterica* and some *E. coli* strains in the 1960s and 1970s (37), but in the mid-1980s it was demonstrated that the linear trisaccharide Kdo2→4Kdo2→4Kdo is present, instead, with the terminal Kdo residue present in non-stoichiometric amounts (38). The reducing Kdo is substituted at O-5 by an α-linked 1-glycero-b-manno-heptopyranose (α1-Hep). The trisaccharide Kdo2→4(α1-Hep1→5)Kdo or, more generally, α2→4(Suga1→5)Kdo represents a common structural element of LPS (11).

The herein reported Kdo tetrasaccharide is only the second to be identified in LPS. The first, having the structure Kdo2→8(Kdo2→4)Kdo2→4Kdo, was identified from a recombinant *E. coli* strain that expressed the Kdo transferase and thus furnished the core region of *C. psittaci* (39).
Interestingly, the Kdo transferase transfers all four Kdo residues and is thus multifunctional. The Kdo transferase(s) of A. baumannii NCTC 10303 has not yet been investigated but that of strain NCTC 15308 was shown to be trifuinctional (14). However, no complete structural analysis of this core region has been undertaken to date. Still the question remains whether one or two different Kdo transferases are present in Acinetobacter. Whereas the former case has been identified in LPS biosynthesis of E. coli (13) and Chlamydia (12), the latter was suggested for LPS of Rhizobium etli (40), in which a third Kdo residue that is substituted by the O-antigen is present at the non-reducing terminus of the core region (41). It was shown that R. etli contains all enzymes necessary to furnish a Kdo disaccharide that is linked to the E. coli lipid A precursor (42). Thus, a single Kdo transferase (as in E. coli) should transfer the first two Kdo residues, whereas a second Kdo transferase was proposed to be responsible for the transfer of the third Kdo residue. The herein reported structure of LPS from A. baumannii NCTC 10303 represents a prerequisite for investigating the question of whether one or more Kdo transferases are needed for core biosynthesis, since it is needed for the construction of defined mutants and/or for chemical synthesis of partial core structures that are employed in the production of monoclonal antibodies.

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