SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

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DEP: 2020 DIRECCION DE NUEVAS CREACIONES **EVE:** 379 FASE NACIONAL INCLUIDO CAPITULO II
TRA: 11 PCT-PATENTE DE INVENCION CAPITULOS I Y II **FOLIOS**: 14
ACT: 519 PRESENTACION

DIRECCIÓN DE NUEVAS CREACIONES

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Bogotá, D.C.,

Abogado(a)/Señor(a)
ANDRÉS MÁRQUEZ ACOSTA

Asunto: Radicación: 11-166480- -6
Trámite: 11
Evento: 379
Actuación: 519
Oficio: 15188
Folios: 14

Patente de Invención	**X**			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	**X**	Capítulo I		Capítulo II	**X**
No. Solicitud Internacional					
Fecha de Presentación Internacional					

Como resultado del examen de fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	No. 11-0166480-00000-0000	02/Diciembre/2011
Reivindicaciones:	No. 11-0166480-00000-0000	02/Diciembre/2011
Dibujos:	No. 11-0166480-00000-0000	02/Diciembre/2011
Prioridad:	P200930265	04/Junio/2009

2. Análisis de la Prioridad

Se acepta la reivindicación de prioridad porque ésta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del capítulo primero del Título 10 de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por el solicitante al momento de comenzar el examen para las reivindicaciones adicionales; esto es que cuando la solicitud contenga más de diez (10) reivindicaciones y no se haya pagado la tasa complementaria, sólo se estudiarán las

cubiertas por la tasa pagada. En el presente capítulo se estudió la totalidad del capítulo reivindicatorio No. 11-0166480-00000-0000 (reivindicaciones 1-21).

Título de la solicitud: “Lipopolisacárido de *Ochrobactrum intermedium* y su uso como inmunoestimulante en mamíferos”.

El problema planteado en esta solicitud consiste en la necesidad de proporcionar lipopolisacáridos para la preparación de un medicamento para el tratamiento y/o prevención de la sepsis, y como adyuvante para vacunas en animales inmunodeprimidos y contra la leishmaniasis.

Para resolver este problema técnico la solicitud en estudio proporciona el aislamiento, la purificación y caracterización de un lipolisacárido de la cepa *Ochrobactrum intermedium* LMG3306.

El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A61K31/739

4. No es una invención (Art 15 D 486 literal (b))

El objeto de la reivindicación 1 no se considera una invención, de acuerdo con el Art citado.

5. Reivindicaciones relativas a un uso o a un segundo uso (Art 14 D 486)

El uso de las reivindicaciones 10-21 no es patentable, de acuerdo con el Art 14.

6. De la solicitud de patente

Descripción (Art 28 D 486)

Debido a que la solicitud se refiere a material biológico, es necesario presentar el Certificado de Depósito para la cepa *Ochrobactrum intermedium* LMG3306.

Titulo

El título no corresponde con el objeto de la solicitud porque los usos no son patentables, por lo cual se sugiere al solicitante hacer la corrección necesaria.

Reivindicaciones (Art 30 D 486, Art 22 PCT)

1- La reivindicación 2 no es clara, debido a que reclama un procedimiento que no está definido por sus etapas. La reivindicación 4 reclama etapas adicionales al procedimiento de la reivindicación 2, sin embargo la reivindicación 2 no menciona ninguna etapa. Se le recuerda al solicitante que un procedimiento se caracteriza por una secuencia lógica de etapas y las condiciones de las mismas.

2- La reivindicación 2 presenta una falsa dependencia de la reivindicación 1 que define el lipolisacárido mientras que la reivindicación 2 define un procedimiento.

3- La composición de las reivindicaciones 5-9 debe ser caracterizada a partir de los elementos que la conforman y las proporciones de los mismos y eliminar las caracterizaciones a partir del uso de esta. El lipopolisacárido debe ser definido por su origen y por su estructura

4- El término “opcionalmente” de la reivindicación 5 no define con claridad el objeto que se desea proteger. Se sugiere reemplazar el término por los excipientes que están divulgados en la descripción y que fueron probados.

Se requiere al solicitante hacer las aclaraciones necesarias.

7. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: Junio 04 de 2009, y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D1	Velasco et al., 1998. Carbohydrate Research. Vol. 306: 1–2, Págs. 283–290	“Structural studies on the lipopolysaccharide from a rough strain of Ochrobactrum anthropi containing a 2,3-diamino-2,3-dideoxy-d-glucose disaccharide lipid A backbone”	1998
D2	Velasco et al., 1998. Int J Syst Bacteriol. Jul; 48 Pt 3:759-68.	“Evaluation of the relatedness of Brucella spp. and Ochrobactrum anthropi and description of Ochrobactrum intermedium sp. nov., a new species with a closer relationship to Brucella spp”	1998
D3	US5824310	“Lipopplysaccharide conjugate vaccines”	1998

El documento D1[1] divulga el aislamiento de un lipopolisacárido de la especie *Ochrobactrum anthropi* (Resumen).

El documento D2[2] divulga la reclasificación de *Ochrobactrum anthropi* a *Ochrobactrum intermedium* (Tabla 1).

El documento D3[3] divulga el uso de un lipolisacárido de la especie Brucella abortus como adyuvante en vacunas para humanos y animales (Resumen).

8. Examen de Patentabilidad (Art 14 D486)

Nivel inventivo (Art18 D486)

La reivindicación 2 consiste en *“un método para la obtención del lipopolisacárido, que comprende el cultivo de la bacteria de la cepa O. intermedium con extracto de soya”*

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D1	D2
Método para la obtención del lipopolisacárido	Método para la obtención del lipopolisacárido de O. anthropi de la cepa LMG 3301	Menciona la reclasificación de Ochrobactrum anthropi a Ochrobactrum intermedium (Tabla 1).
Cultivo de la cepa O. intermedium con extracto de soya	Cultivo de la cepa con extracto de soya (Sección 4.1).	

El documento D1 se considera el estado de la técnica cercano a la invención definida en la reivindicación 2 porque divulga un método para la obtención del lipopolisacárido

1. http://www.sciencedirect.com/science/article/pii/S0008621597100295

2. http://ijs.sgmjournals.org/content/48/3/759.long

3.http://worldwide.espacenet.com/publicationDetails/description?CC=US&NR=5824310A&KC=A&FT=D&ND=&date=19981020&DB=&locale=en_EP http://ijs.sgmjournals.org/content/48/3/759.long

de O. intermedium de la cepa LMG 3301 comprendiendo el cultivo de la cepa, la centrigugación, lavado con acetona y posterior liofilización (Sección 4.1). El documento D2 divulga la reclasificación de *Ochrobactrum anthropi* a *Ochrobactrum intermedium* (Tabla 1).

La diferencia entre la invención, definida en la reivindicación 2 y el procedimiento de D1 consiste en que el lipopolisacárido es extraído de la cepa LMG 3301.

El efecto que logra el incluir que el lipopolisacárido es extraído de la cepa LMG es que se obtiene un lipopolisacárido de la cepa LMG3306.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención consiste en la necesidad de un método, para para obtener un lipopolisacárido de una cepa alternativa de O. intermedium, al ya conocido en el documento D1.

Sin embargo, teniendo en cuenta que el documento D1 ya divulgaba un método para la obtención de un lipolisacárido de la cepa LMG 3301 y que la cepa LMG 3306 ya estaba divulgada en D2 que menciona la reclasificación de Ochrobactrum anthropi a Ochrobactrum intermedium, la persona del oficio medianamente versada en la materia habría inferido sin mucho esfuerzo de su parte, obtener lipopolisacáridos de otra cepa usando el método de D1.

Conclusión:
La reivindicación 2 cumple el requisito de novedad (Art. 16), pero no cumple con el requisito de nivel inventivo (Art. 18).

La reivindicación 5 consiste en *"una composición inmunoestimulante que comprende el lipopolisacárido de Ochrobactrum intermedium LMG3306 y opcionalmente uno o más excipientes farmacéuticamente aceptables".*

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	**D2**	**D3**
Composición	No menciona	Vacuna
Lipolisacárido de la especie *Ochrobactrum intermedium*	Menciona la cercanía entre la especie O. intermedium y brucellae (Pág. 48, Col. 2)	Lipopolisacárido de la especie *Brucella abortus* (Resumen de la invención)
Excipiente farmacéuticamente aceptable	No menciona	Vehículo farmacéuticamente aceptable (Resumen de la invención)

El documento D3 se considera el estado de la técnica cercano a la invención definida en la reivindicación 5 porque divulga una vacuna que comprende un lipopolisacárido de la especie *Brucella abortus* con un vehículo farmacéuticamente aceptable (Resumen de la invención).

La diferencia entre la invención, definida en la reivindicación 1 y la vacuna de D3 consiste en que el lipopolisacárido es extraído de la especie *Brucella abortus* en vez de la especie *Ochrobactrum intermedium.*

Esta diferencia no parece conceder un efecto técnico inesperado o una ventaja a la vacuna reclamada en esta solicitud.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención consiste en la necesidad de una vacuna alternativa a los ya conocida en el documento D3.

Teniendo en cuenta que el documento D3 ya divulgaba vacunas que comprenden

lipolisacáridos y un vehículo farmacéuticamente aceptable y que el documento D2 divulga la cercanía entre la especie O. intermedium y brucellae (Pág 48, Col. 2), la persona del oficio medianamente versada en la materia habría inferido sin mucho esfuerzo de su parte, obtener lipopolisacáridos de otras especies para obtener composiciones inmunoesimulantes.

Conclusión:
La reivindicación 5 cumple el requisito de novedad (Art. 16), pero no cumple con el requisito de nivel inventivo (Art. 18). Las reivindicaciones 6-9 tampoco cumplen con el requisito de nivel inventivo.

9. Conclusión

Los requisitos de Patentabilidad de la presente solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	Velasco et al., 1998. Carbohydrate Research. Vol. 306: 1–2, Págs. 283–290	2	Nivel inventivo
D2	Velasco et al., 1998. Int J Syst Bacteriol. Jul; 48 Pt 3:759-68.	2, 5-9	Nivel inventivo
D3	US5824310	2	Nivel inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Finalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones, no pagará la tasa de modificación voluntaria pero sí la correspondiente al pago por reivindicación adicional.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.

JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (c)

El anterior requerimiento fue notificado por fijación en lista, de fecha 2013-08-20

Elaboró: Lina Maria Lizaraso Trespalacios
Revisó: Jeny Paola Villate Becerra

ELSEVIER

Carbohydrate Research 306 (1998) 283–290

CARBOHYDRATE RESEARCH

Structural studies on the lipopolysaccharide from a rough strain of *Ochrobactrum anthropi* containing a 2,3-diamino-2,3-dideoxy-D-glucose disaccharide lipid A backbone

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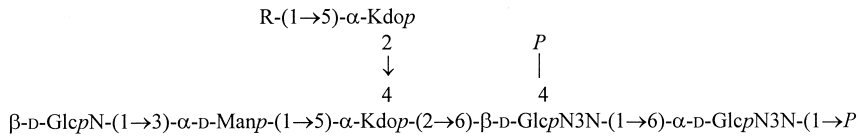
Abstract

A degradation protocol using de-*O*-acylation and subsequent alkaline de-*N*-acylation was applied to the lipopolysaccharide of *Ochrobactrum anthropi* rough strain LMG 3301. Three main oligosaccharide bisphosphates containing core–lipid A backbone structures were obtained after fractionation by anion-exchange HPLC. Using ^{1}H and ^{13}C NMR spectroscopy, including two-dimensional COSY, TOCSY, and NOE spectroscopy (ROESY and NOESY), the following structures were established:



where Kdo is 3-deoxy-D-*manno*-octulosonic acid, D-GlcN3N is 2,3-diamino-2,3-dideoxy-D-glucose and R is H or α-D-Gal*p*A or 4-deoxy-β-L-*threo*-hex-4-enopyranuronic acid, the latter sugar being derived from α-D-Gal*p*A by β-elimination of a substituent attached to O-4. This is the first report on the isolation from a lipopolysaccharide of an oligosaccharide containing

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GlcN3N in the lipid A backbone [β-D-Glc*p*N3N4*P*-(1 → 6)-α-D-Glc*p*N3N1*P*]. Sugar and methylation analysis confirmed the presence of the GalA → Kdo disaccharide and non-stoichiometric substitution of GalA. It is suggested that Glc is the substituent at O-4 in GalA and that in the non-degraded lipopolysaccharide the amino group of GlcN is not acylated.

Keywords: Lipopolysaccharide; Core–lipid A backbone; 2,3-Diamino-2,3-dideoxy-D-glucose; Alkaline degradation; NMR spectroscopy; *Ochrobactrum anthropi*

1. Introduction

Ochrobactrum anthropi is a Gram-negative bacterial species which includes isolates from immunocompromised patients, environmental and hospital water sources, as well as from nematodes, suggesting that the original habitat could be soil (see references cited in ref. [1]). Molecular taxonomy studies place *O. anthropi* within the α-2 subgroup of the class Proteobacteria showing the greatest relatedness with *Brucella*, an important intracellular parasite of animals and humans [2].

Lipopolysaccharide (LPS, endotoxin) of the outer membrane of Gram-negative bacteria is important in pathogenicity, and a link between some properties of the *Brucella* LPS and the ability to multiply intracellularly has been shown [3–5]. Therefore, a molecular comparison of the *Brucella* and *O. anthropi* LPS could contribute to a better understanding of the pathogenicity of these bacteria. The structures of the O-specific polysaccharides and lipid A of the *Brucella* LPS have been characterised [6–8]. Recently, the structure of the O-specific polysaccharide of the reference strain *O. anthropi* LMG 3331 has been elucidated [1]. Now, we report on structural studies of the core and lipid A backbone of the LPS from a rough strain *O. anthropi* LMG 3301 and on the isolation, for the first time, of oligosaccharides with a GlcN3N lipid A backbone structure.

2. Results

The LPS was isolated from dried bacterial cells of rough *O. anthropi* strain LMG 3301 by the phenol–chloroform–light petroleum procedure [9]. Tricine–SDS–PAGE showed that the LPS obtained was similar to those produced by enterobacterial R-strains and revealed a structural heterogeneity with a major compound migrating at the front and two additional bands (Fig. 1).

The LPS was de-*O*-acylated by mild hydrazinolysis and de-*N*-acylated by strong alkaline hydrolysis according to the published protocol [10,11]. Fractionation of the resulting carbohydrates by anion-exchange HPLC on CarboPac PA-1 afforded three main oligosaccharide products designated as OS1, OS2, and OS3.

All oligosaccharides contained 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) and phosphate. Sugar analysis using GLC–MS of acetylated methyl glycosides derived after *N*-acetylation, revealed the presence of Man, GlcN, 2,3-diamino-2,3-dideoxyglucose (GlcN3N), and Kdo. In addition, OS2 contained GalA. Man and GlcN were additionally identified by GLC of acetylated alditol acetates.

The ^{1}H NMR spectrum of OS2 (Fig. 2) contained signals for two 3-deoxy groups (H-3) at δ 2.13 and 2.10 (equatorial) and δ 2.01 and 1.83 (axial) typical of α-pyranoid Kdo [10] and six signals in the resonance region of anomeric protons, one of which belongs to H-1 of an α-glycosyl phosphate (δ 5.43, $J_{1,2}$ 3 Hz, $J_{\text{H-1,P}}$ 8 Hz), one to an α-linked pyranose (δ 5.23, $J_{1,2}$ 3.5 Hz), and two to β-linked pyranoses (δ 4.55 and 4.60, $J_{1,2}$ 8–8.5 Hz) with axial H-2. One more doublet at δ 5.30 ($J_{1,2}$ 2 Hz) was assigned to H-1 of a pyranose with equatorial H-2 (Man) and the remaining signal at δ 4.73 ($J_{4,5}$ 1 Hz) to H-5 of GalA (Table 1). Signals for other sugar protons were found in the region δ 2.70–4.32.

The ^{1}H NMR spectrum of OS2 was completely assigned using two-dimensional (2D) gradient selected COSY, double quantum filtered (DQF) COSY (Fig. 2), and TOCSY (Table 1). On the basis of the chemical shifts and coupling constant values, the seven sugar spin systems were ascribed to α-Man*p*, α-Gal*p*A, β-Glc*p*N, α-Glc*p*N3N1*P*, β-Glc*p*N3N4*P*, and two α-Kdo*p* residues (Kdo^{I} and Kdo^{II} enumerated starting from the reducing end of the oligosaccharide, see the structure of OS2 shown below). The assignment of the spin systems of the amino sugars was confirmed by correlation of the protons at the carbons bearing nitrogen (H-2 of GlcN and H-2

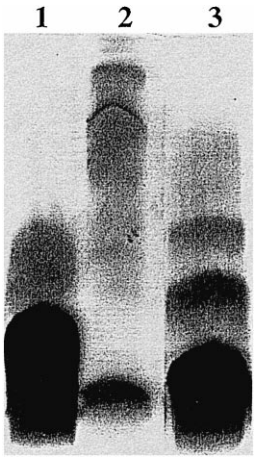


Fig. 1. Silver-stained Tricine–SDS–PAGE of LPSs from (1) *E. coli* F515 (R-type), (2) *O. anthropi* LMG 3331 (S-type), and (3) *O. anthropi* LMG 3301 (R-type).

and H-3 of GlcN3N, δ_H 2.70–3.18) to the corresponding carbons in the region δ_C 53–59 observed in the H-detected $^1H,^{13}C$ HMQC spectrum of OS2. The attachment of the second phosphate group to O-4 of β-GlcN3N followed from a low-field position of the signal for H-4 at δ 3.78, e.g., as compared with δ 3.40 for H-4 in β-GlcN, and from its coupling to phosphorus ($J_{H\text{-}4,P} \sim 10$ Hz).

Linkage and sequence analysis of OS2 was performed using 2D rotating-frame NOE spectroscopy (ROESY). As expected, α-GlcN3N H-1 exhibited no interresidue correlation, and β-GlcN3N H-1 gave a strong cross-peak with α-GlcN3N H-6b at δ 4.55/3.73. This confirmed that the two diamino sugar residues build up the lipid A backbone of the LPS and, therefore, OS2 is a product of the expected type [10,11], containing a core oligosaccharide (or its part) attached to a bisphosphorylated lipid A backbone [β-D-Glc*p*N3N4*P*-(1 → 6)-α-D-Glc*p*N3N1*P*].

Two inter-residue cross-peaks of similar intensity, with Man H-2 and H-3, were observed for GlcN H-1 at δ 4.60/4.32 and 4.60/4.15, respectively, that with the same absolute configurations of both monosaccharides and the β-glycosidic linkage, strongly indicated that Man is 3-substituted by GlcN [12].

Each of Man H-1 and GalA H-1 exhibited two inter-residue correlations, with both H-5 and H-7 of Kdo^I (Man, δ 5.30/4.27 and 5.30/3.72, respectively) and Kdo^{II} (GalA, δ 5.23/4.12 and 5.23/4.15), thus, indicating the α-(1 → 5) linkage between the two monosaccharide pairs (cf. published data for an α-(1 → 5)-linked heptose-Kdo disaccharide [13]). A NOE between Kdo^{II} H-3ax and GalA H-5 observed at δ 1.83/4.73 was consistent with the structure proposed. Such a NOE is characteristic for this linkage and has been repeatedly described for Sug-(1 → 5)-Kdo disaccharides [13,14].

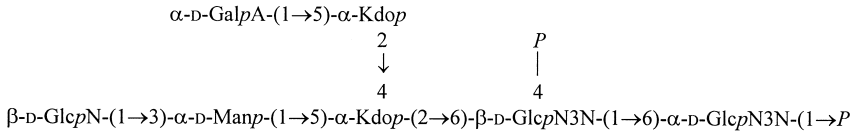
The connectivities between Kdo^I H-3eq,3ax and Kdo^{II} H-6 at δ 2.10/3.71 and 2.01/3.71 proved the α-(2 → 4)-linkage between the two Kdo residues [15]. Finally, the attachment of Kdo^I to β-GlcN3N was confirmed by the characteristic chemical shifts for H-5, H-6a, and H-6b of the latter sugar, δ 3.73, 3.76, and 3.47, respectively [cf., e.g., the corresponding chemical shifts δ 3.70, 3.71, and 3.51 for β-GlcN in the de-*N*,*O*-acylated LPS from *Salmonella minnesota* R595 [10] but δ 3.49, 3.86, and 3.78 in the nonsub-

Table 1
500 MHz ^1H NMR data for OS2 (δ in ppm)

	H-1	H-2	H-3eq/H-3ax	H-4	H-5	H-6a/H-6b	H-7	H-8a/H-8b
β-D-Glc*p*N-(1 →	4.60	2.70	3.45	3.40	3.53	3.93 3.73		
→ 3)-α-D-Man*p*-(1 →	5.30	4.32	4.15	3.89	4.06	3.88 3.88		
α-D-Gal*p*A-(1 →	5.23	3.85	3.98	4.30	4.73			
→ 5)-α-Kdo p^{II}-(2 →			2.13 1.83	4.12	4.12	3.71	4.15	4.00 3.76
→ 5)-α-Kdo p^{I}-(2 → 4 ↑			2.10 2.01	4.14	4.27	3.75	3.72	3.90 3.71
→ 6)-β-D-Glc*p*N3N-(1 →	4.55	2.78	3.16	3.78	3.73	3.76 3.47		
→ 6)-α-D-Glc*p*N3N-(1 →	5.43	2.95	3.18	3.55	4.13	4.30 3.73		

stituted enterobacterial lipid A backbone (Zähringer, unpublished data)].

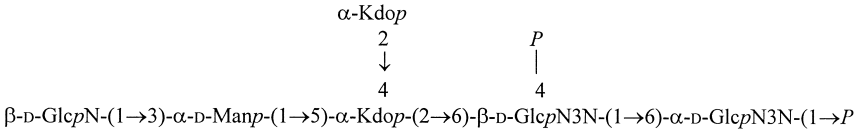
Therefore, on the basis of these data, it was concluded that OS2 has the following structure:



OS2

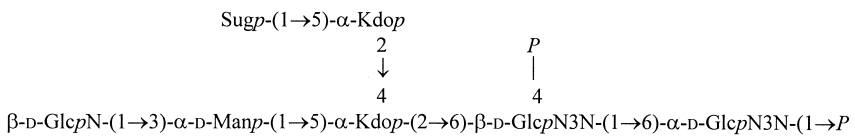
Since substitution of Kdo^{II} at position 5 is not common [16], this was further confirmed by methylation analysis of the GalA-(1 → 5)-Kdo disaccharide released by mild acid degradation of the LPS. The structure of the reduced and methylated disaccharide was identified by the mass spectrum which contained peaks of typical fragment ions at *m/z* 233 (the GalA moiety) and 307 (the reduced Kdo moiety). After methanolysis of the methylated disaccharide and acetylation, GLC–MS analysis revealed methyl 5-*O*-acetyl-3-deoxy-2,4,6,7,8-penta-*O*-methyloctanoate identified by comparison with the authentic sample, thus strongly demonstrating 5-substitution of Kdo.

Similar NMR studies were carried out with OS1 and OS3 and confirmed that both lack GalA. Comparison of the ^{1}H NMR spectra of OS1 and OS2 demonstrated that the absence of GalA from the former is the only difference between the oligosaccharides. In particular, the following characteristic signals listed above for OS2 had essentially the same chemical shifts in OS1: δ 5.45 (H-1, α-GlcN3N), 5.30 (H-1, Man), 4.64 (H-1, GlcN), 4.54 (H-1, β-Glc3N), 2.15 (H-3eq, Kdo^{II}), 2.10 (H-3eq, Kdo^{I}), 2.02 (H-3ax, Kdo^{I}), 1.80 (H-3ax, Kdo^{II}), while no signals for H-1 and H-5 of GalA were present in the spectrum. Hence, OS1 has the following structure:



OS1

In OS3, instead of the signals for GalA, the ^{1}H NMR spectrum revealed signals for a 4-deoxy-β-L-*threo*-hex-4-enuronic acid (Sug) at δ 5.74 (H-4, $J_{3,4}$ 3 Hz), 5.34 (H-1, $J_{1,2}$ 2.5 Hz), 4.40 (H-3, $J_{2,3}$ 7.5 Hz), and 3.80 (H-2) (cf. published data [17]) together with essentially the same chemical shifts of the characteristic signals listed above for OS1 and OS2. The unsaturated sugar has been reported to derive from a 4-substituted hexuronic acid by β-elimination during strong alkaline treatment [17]. Like GalA in OS2, Sug in OS3 is attached at position 5 of Kdo^{II}, as followed from a 2D NOESY experiment which revealed strong cross-peaks Sug H-1, Kdo^{II} H-5 and H-7 at δ 5.34/4.25 and 5.34/4.04, respectively. The 2D COSY and NOESY patterns displayed by OS2 and OS3 were similar and, therefore, OS3 has the following structure:



OS3

To shed light on the nature of the substituent at O-4 of GalA, sugar and methylation analyses of the native LPS were performed. Neutral sugars derived from LPS by acid hydrolysis were Glc and Man, the latter being detected in only trace amounts. Most likely, this was due to the absence of any acyl group

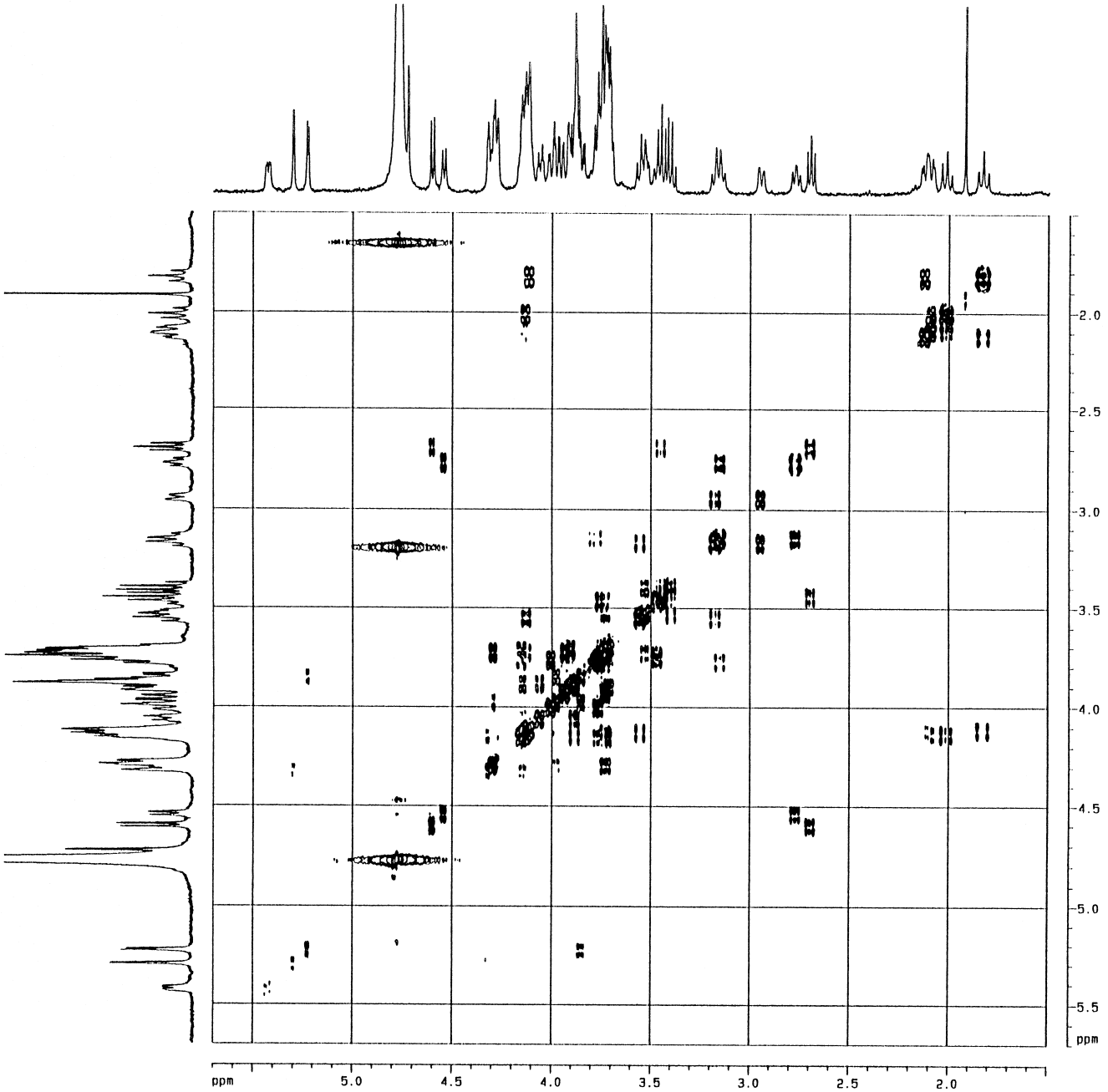


Fig. 2. 500 MHz 2D DQF COSY spectrum of OS2. The corresponding 1D ^{1}H NMR spectrum is displayed along the axes.

at the amino group of GlcN that makes the linkage between GlcN and Man stable to acid hydrolysis [18]. When methanolysis and carboxyl reduction using $NaBD_4$ were performed prior to hydrolysis and the second reduction with $NaBD_4$, almost equal amounts of glucitol-1-*d* and galactitol-1,6,6-d_3 were identified, the latter resulting evidently from GalA. Methylation of LPS followed by hydrolysis and GLC–MS of derived alditol acetates revealed only terminal Glc, since from the other terminal sugar residues (Kdo, GalA, GlcN) suitable derivatives are not obtained by this procedure and GlcN–Man was only detected as a disaccharide. After methanolysis and acetylation GLC–MS analysis indicated the presence of terminal GalA and monosubstituted GalA. These data fitted well with the structures of OS2 and OS3 and, therefore, it can be suggested that in part of the LPS species Glc is attached to GalA at position 4.

3. Discussion

This study is the first successful application of a recently elaborated strong alkaline degradation protocol [10,11] to a LPS containing a GlcN3N disaccharide lipid A backbone, demonstrating that this method is not limited by the enterobacterial-type GlcN disaccharide lipid A backbone [β-D-Glc*p*N3N4*P*-(1 → 6)-α-D-Glc*p*N3N1*P*]. This finding is important in view of the increasing number of bacteria recognised recently to contain a lipid A backbone of the former type [8,19–21].

SDS–PAGE revealed structural heterogeneity in the *O. anthropi* LPS which may be associated with different substitution in Kdo^{II}: this may be unsubstituted (OS1) or substituted with GalA (OS2) or with a longer fragment containing GalA substituted at O-4. The structure of the substituent could not be determined using the strong alkaline protocol because of β-elimination in GalA resulting in a truncated oligosaccharide (OS3). It was suggested that this substituent is a glucose residue, but further studies are necessary to determine the complete structure of the core region of the *O. anthropi* strain LMG 3301 LPS.

Lipid A and the inner core are conserved parts of LPS within the same serogroup and their structures are considered as a marker of the phylogenetic relatedness among Gram-negative bacteria [19]. Therefore, demonstration of a GlcN3N-based lipid A backbone in *O. anthropi* is in keeping with the finding of such lipid A backbone in LPS of other members of the α-subgroup of Proteobacteria [5,8,21], including *Brucella* [5,8].

The inner core of *O. anthropi* includes a Kdo disaccharide but lacks L-*glycero*-D-*manno*-heptose, which is typically linked to Kdo^{I} in most LPS, the structures of which were elucidated. This is the case also for the LPSs of *Brucella* [22] and some *Mycoplana* spp. [21], including *M. dimorpha* which forms a close cluster with *Brucella* and *O. anthropi* [23]. The LPS of all these bacteria contain Man, which in *O. anthropi* occupies the same position as would be expected for heptose; location of Man in *Brucella* and *Mycoplana*, as their core structures as the whole, remains unknown. It is worth noting that the inner core and lipid A backbone structures of the *O. anthropi* LPS resemble much those of *Legionella pneumophila* serogroup 1 (strain Philadelphia 1) [20,24], both bacteria containing the same bisphosphorylated GlcN3N disaccharide in lipid A and the same α-D-Man-(1 → 5)-Kdo^{I} disaccharide and no phosphate in the core.

4. Experimental

Bacterium, growth and isolation of LPS.—*O. anthropi* strain LMG 3301 was from the culture collection of the Laboratory of Microbiology, Gent University, Belgium. The bacterium was grown in a fermenter and harvested by centrifugation as described previously [1]. The sedimented bacterial cells were washed successively with EtOH, acetone (twice) and Et_2O, and then dried in vacuum. LPS was extracted from the dried cells with 2:5:8 phenol–chloroform–light petroleum [9] in a yield of 3% of the bacterial dry mass.

SDS–PAGE.—SDS–PAGE was performed using a discontinuous buffer system with Tris–Tricine buffers [25]. A total of 10 μg of each LPS from *Escherichia coli* strain F515, *O. anthropi* strain LMG 3331, and *O. anthropi* strain LMG 3301 were applied to the gel. The gel was fixed and silver-stained [26].

NMR spectroscopy.—The ^{1}H and ^{13}C NMR spectra were recorded with Bruker DRX 600 and Bruker DRX 500 spectrometers for solutions in D_2O at pD 7 at 300 K using internal sodium 3-trimethylsilylpropanoate-d_4 (δ_H 0.00) as reference. 2D NMR experiments were performed using standard Bruker software. Mixing times of 70, 250, and 150 ms were used in TOCSY, ROESY, and NOESY experiments, respectively.

Chromatography and GLC–MS.—GPC was performed on a column (120 × 2.5 cm) of Sephadex G-10 in water or a column (60 × 2.5 cm) of Sephadex G-50, and monitored using a Knauer differential refractometer. Anion-exchange HPLC was carried out on a column (250 × 9 mm) of CarboPac PA-1 at 4 mL/min in a gradient of NaOAc (0–0.7 M) in 0.1 M NaOH for 40 min with monitoring using a pulse amperometric detector (Dionex, USA). GLC was performed on a Varian 3700 chromatograph equipped with a fused-silica gel SPB-5 column using a temperature gradient 150 °C (3 min) → 320 °C at 5°C/min. GLC–MS was performed on a Hewlett-Packard 5989A instrument equipped with an HP-5 column under the same chromatographic conditions as in GLC.

De-O-acylation and alkaline degradation of LPS.—LPS (700 mg) was treated with anhydrous hydrazine (0.5 h, 37 °C) [10], and the resulting de-*O*-acylated LPS (566 mg) was de-*N*-acylated with 4 M KOH (16 h, 120 °C). Then the mixture was neutralised with 4 M HCl, fatty acids were extracted with CH_2Cl_2 (twice), the aqueous phase was desalted by GPC on Sephadex G-10, and the product (125 mg)

was fractionated by anion-exchange HPLC. Desalting by GPC of the three major fractions eluted with a retention time of 28.5, 30, and 36 min afforded OS1 (4.8 mg), OS2 (1.8 mg), and OS3 (5.6 mg), respectively.

Sugar and phosphate analyses.—Oligosaccharides (0.3 mg each) were *N*-acetylated with Ac_2O in aq $NaHCO_3$ as described [11], then hydrolysed with 2 M CF_3COOH (4 h, 100 °C) or methanolysed with 2 M HCl in MeOH (16 h, 85 °C). LPS was hydrolysed as above without or with prior methanolysis and carboxyl-reduction with $NaBD_4$ and the products were reduced with $NaBD_4$. Samples were analysed by GLC and GLC–MS after conventional acetylation with Ac_2O in pyridine. Absolute configurations of monosaccharides were determined by GLC of acetylated (+)-2-butyl glycosides [27,28]. Kdo was determined by the thiobarbituric acid reaction according to the modified method [29]. Phosphate was determined by the method of Lowry et al. [30].

Methylation analysis.—LPS was degraded with aq 2% HOAc (3 h, 100 °C), the water-soluble portion was fractionated by GPC on Sephadex G-50, and the low-molecular-mass fraction (a GalA → Kdo disaccharide) was reduced with $NaBH_4$ and methylated [31]. A portion of the methylated material was methanolysed with 2 M HCl in MeOH (85 °C, 4 h) and acetylated (Ac_2O–pyridine, 20 min, 85 °C). LPS was methylated [31], hydrolysed and reduced with $NaBH_4$ or methanolysed as in sugar analysis. Both methylated disaccharide and monosaccharides were analysed by GLC–MS.

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