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(54) Title of the Invention:

# LIPOPOLYSACCHARIDES EXTRACTED FROM *ESCHERICHIA COLI*

(57) Abstract:

The invention concerns lipopolysaccharides extracted from *E. coli* DSM 6601. This LPS differs from previously known LPS from *E. coli* especially with respect to the phosphorylated sugar moiety of the core and the degree of polymerization of the O-specific chain. The lipid A corresponds structurally and biologically to the usual type for *E. coli*. The LPS described here not only is well suited for identifying the coli strain that carries

it, but also reduces the pathogenicity of the coli strain while allowing it to retain its immunomodulatory effect.

## LIPOPOLYSACCHARIDES EXTRACTED FROM *ESCHERICHIA COLI*

The invention concerns new lipopolysaccharides extracted from *E. coli*.

Endotoxins are bacterial structural components, which, unlike exotoxins, are not secreted, but rather are released, especially following autolysis. The classic endotoxins are heat-stable lipopolysaccharides (LPS) from the outer cell membrane of gram-negative bacteria. LPS consists of lipid A, which is responsible for the toxic effect of LPS, a core oligosaccharide and an O-specific chain.

In macroorganisms, endotoxins stimulate the production of immune system mediators, such as interleukin-1 (IL-1) and tumor necrosis factor (TNFα).

Many studies have already been conducted on the composition of the endotoxins of enterobacteria, especially *E. coli*, in which is was determined that S/R mutants generally contain only one repeating unit of their O-specific chain (cf. Figure 1). It is assumed that in these cases, the gene that codes for the polymerizing enzyme of the O-specific chain is defective, and therefore only one repeating unit it transferred to the core oligosaccharide. LPS structures of a similar type but different structure are also commonly found in bacteria that are pathogenic in man, such as *Neisseria*, *Vibrio*, *Campylobacter*, *Helicobacter*, etc. These bacteria have an LPS which allows them to evade the immune defense of the host by means of a special molecular mimicry, including the presence of sialic acid and oligosaccharides that contain sialic acid, which resemble glycoproteins and glycolipids in mammals. The O6 serotype was determined for *E. coli* DSM 6601. This structure was studied and published by P. E. Jansson et al., *Carbohydr. Res.* 131 (1984) 277-283. The structure corresponds to the formula shown in Figure 2.

The lipid A of the coli bacteria has also been investigated by various research groups, and it was found that the structure of the lipid A generally has the hexaacyl form and is consistent for all serotypes of *E. coli* (Figure 3). The structure of the hexaacyl compound was published in 1984 by T. Rietschel et al., Structure and Conformation of the Lipid A Component of Lipopolysaccharides. *Handbook of Endotoxins* (Proctor, R., ed.), Vol. 1, *Chemistry of Endotoxin* (E. T. Rietschel, ed.), Elsevier, Amsterdam (1984), pp. 187-220. The structure is shown in Figure 3.

The O-specific chain and the lipid A are linked by the core oligosaccharide. There are five previously known core oligosaccharides of *E. coli*; see O. Holst et al., Chemical Structure of the Core Region of Lipopolysachharide, IN: *Bacterial Endotoxic Lipopolysaccharides*, Vol. 1, Morrison, D. C. and Ryan, J. L. (eds.), Boca Raton, FL, USA (1992) pp. 135-170 (cf. Figure 4).

Studies of the LPS of *E. coli* strain DSM 6601 revealed that the composition of the lipid A corresponds to the hexaacyl form of the lipids A otherwise described for *E. coli*.

The studies with respect to the release of IL-1 and TNFα in human monocytes confirm that this lipid A has the same activity as *E. coli* lipid A and therefore very probably corresponds in its structure to the known structure of *E. coli* lipid A (cf. Figure 5, Figure 6). This assumption was confirmed by the chemical analyses.

The structure of the specific O antigen of *E. coli* DSM 6601 is surprising due to the fact that apparently only a single repeating unit is normally present in the chain (cf. Figure 1), which leads to the conclusion that the strain DSM 6601 is an S/R mutant, which is extremely unusual for a human isolate. However, as the serologic analysis shows, the structure of this repeating unit corresponds to the basic pattern of the O-specific chain of *E.*

*coli* O 6.

Although the core region in strain DSM 6601 corresponds to the well-known R1 structure, structural peculiarities are present. Specifically, 8 phosphate groups were analytically determined per LPS molecule, and the lipid A generally has only 2 phosphate groups. Furthermore, a nonstoichiometric content of pyrophosphoethanolamine was found.

Therefore, it can be stated in summary that the LPS of the strain DSM 6601 differs significantly from the previously known LPS from *E. coli*, especially with respect to the phosphorylated sugar moiety of the core and the degree of polymerization of the O-specific chain. The lipid A corresponds structurally and biologically to the usual type for *E. coli*. The LPS described here not only is well suited for identifying the coli strain that carries it, but also reduces the pathogenicity of the coli strain while allowing it to retain its immunomodulatory effect. The fact that the O-specific chain is linked by a β-glycosidic bond instead of an α-glycosidic bond could be clearly shown for the first time for *E. coli* with the example of the S/R mutant DSM 6601.

The lipopolysaccharide (LPS) of *E. coli* DSM 6601 is a new smooth-rough (S/R) structure, which, on the one hand, is composed of previously known partial structures (O-specific chain, core oligosaccharide and lipid A) and, on the other hand, was completely structurally characterized for the first time in the complex form that exists here (cf. Figure 7). The O-specific chain, which consists of only a single repeating unit of the serotype O6, is linked to the core oligosaccharide by a β-glycosidic bond, which differs from the linkages within the O-specific chain (α-glycosidic). The core oligosaccharide has the R1 structure, a chemical finding that is confirmed by serologic tests with R1-specific antibodies. The lipid A component has a specific chemical structure that is characteristic of *E. coli* lipid A.

The LPS of *E. coli* strain DSM 6601 exhibits astonishing homogeneity. Heterogeneity can be observed only with respect to the phosphate substituents (PP and P-Etn vs. P and P), which is being described in this form for the first time. The P-Etn substituent could be definitely determined in the core oligosaccharide, the R1-core oligosaccharide, at the 2 position of the second heptose ($Hep^{II}$) by complex NMR analyses.

The complete structure of the LPS of the strain DSM 6601 is shown in Figure 7.

The invention is explained in greater detail below by examples.

## E X A M P L E 1

### PREPARATION OF THE LPS

The LPS was obtained from the washed and dried bacterial mass by a modified phenol/water extraction; for further details on this aspect of the preparation, see O. Westphal et al., Bacterial Lipopolysaccharides, Extraction with Phenol-Water and Further Applications of the Procedure, *Meth. Carbohydr. Chem.*, Vol. V (1965), pp. 83-91.

47 g of the lyophilized bacteria, which had first been washed twice with distilled water, were extracted by a modified method of Westphal and Jann. The modification consisted in a subsequent enzyme treatment (DNase, RNase, proteinase K) of the aqueous extract, the purpose of which was to remove possible foreign proteins and DNA/RNA components. To this end, the aqueous phase (about 1.2 L) is treated at room temperature with 20 mg of RNase (ribonuclease A, bovine pancreas, Sigma) and 20 mg of DNase (DNase I, bovine pancreas, grade II, Sigma). The mixture is stirred for 30 h at room temperature, treated with 20 mg of proteinase K (*Tritirachium album*, Boehringer, Mannheim), and stirred for another 12 h. The suspension is

dialyzed three times against 15 L of distilled water over 24 h at 4°C and then lyophilized. The enzyme-treated extract is resuspended in distilled water to an end concentration of 50 mg/mL. This suspension is ultracentrifuged three times at low temperature (155,000 × g, 4°C, 4 h). The sediment (LPS) is suspended in 150 mL of distilled water, dialyzed again for three days against water, and then lyophilized (yield of LPS: 1.45 g, 3.1% m/m).

## E X A M P L E 2

### ANALYSIS OF THE LPS EXTRACTED FROM *E. COLI* STRAIN DSM 6601

Hexosamine (HexN) (meaning here glucosamine + galactosamine, GlcN + GalN) was determined by the modified Morgan-Elson test (Strominger, J. L., Park, J. T., Thompson, R. E., *J. Biol. Chem.* 234, 3263-3268 (1959)) or alternatively by HPLC (PICO-TAG, Waters). In contrast to the Morgan-Elson test, in this analytical method, it is possible not only separately to determine and quantify GlcN and GalN, but also to make parallel determinations of the presence of GlcN phosphate, 2-ethanolamine (Etn) and 2-ethanolamine phosphate (Etn-P), which often occur in LPS. Gas-liquid chromatography (GC) was performed in a Varian 3700 GC or Hewlett Packard (HP 5890 Series II) gas chromatograph on a capillary column (fused-silica SPB-5®, 30 m, Supelco). The combined gas-liquid chromatography/mass spectrometry (GC-MS) was performed in a mass spectrometer (HP model 5989) equipped with an HP-1 capillary column (30 m, Hewlett Packard). The GC and GC-MS analyses were used to determine the neutral sugars (Glc, Gal, Hep, Man) as their alditol acetates (Sawardeker, J. S., Slonerker, J. H., Jeanes, A., *Anal. Chem.* 37, 1602-1604 (1967)) and to determine and quantify the fatty acids as their fatty acid methyl ester derivatives after intense methanolysis (2 *M* HCl/MeOH, 120°C, 16 h) (Wollenweber, H.-W. and Rietschel, E. T., Analysis of Lipopolysaccharide

(Lipid A) Fatty Acids, *J. Microbiol. Meth.* 11, (1990) 195-211) and extraction with chloroform. In both GC analytical methods, the initial temperature was 150°C (isothermal for 3 min), and then the temperature was increased to 320°C by a linear temperature gradient of 5°C/min. Phosphate was determined by the method of Lowry et al. (Lowry, O. H., Roberts, N. R. , Leiner, K. Y., Wu, M. Kl., Farr, A. L., *J. Biol. Chem.* 207, 1-17 (1954)), and the 2-keto-3-deoxy-D-manno-octulosonic acid (Kdo) was determined by the thiobarbituric acid test (Waravdekar, V. C. and Saslaw, L. D., *J. Biol. Chem.* 234, 1945-1950 (1959)).

## PREPARATION AND PURIFICATION OF THE FREE LIPID A AND THE CORE OLIGOSACCHARIDE

LPS (258.8 mg) was suspended in 25 mL of 0.1 *M* NaOAc/HOAc (ph 4.4) and subjected to gentle acid hydrolysis at 100°C for 1 h. The lipophilic fraction (lipid A) was then extracted three times from the hydrolysate with 25 mL of chloroform (yield: 23.2 mg). The lipid A from the organic phase was further purified by preparative thin-layer chromatography (PTLC) (2 mm PTLC silica gel 60 plate, E. Merck, Darmstadt), which was chromatographed with chloroform-methanol-water 100:75:15 (v/v/v) and developed by immersion in distilled water. In this way, six fractions were obtained, of which the principal fraction ($R_f \approx 0.4$) represents the purified diphosphorylated hexaacyl lipid A (DPHLA-$EC_{6601}$). The purified DPHLA-$EC_{6601}$ (yield: 2.06 mg) was dissolved in chloroform-methanol 8:2 (v/v) and treated with ion exchanger (Amberlite IRA 120, $H^+$ form) before the MALDI-TOF-MS. An aliquot portion (250 µg) of the purified DPHLA-$EC_{6601}$ was used for the biological experiments.

The aqueous phase from the chloroform extraction was lyophilized (yield: 272 mg), and the oligosaccharide was further purified by means of a TSK column [3.5 × 90 cm, TSK HW-40(S), E. Merck] in pyridine-acetic acid-water 8:20:2000

(v/v/v). The individual oligosaccharide fractions (pool A, B, C, D) were analyzed by GC-MS and NMR spectroscopy. The principal fraction (pool A, #28-41; 49.05 mg), which contained both sugar components of the O-specific chain (Man, GalNac) and sugar components of the core oligosaccharide (Hep, Kdo), was further purified. The other fractions contained monosaccharides, artifacts of Kdo (anhydro- and lactones), which were not further analyzed, and, finally, salt. The principal fraction of the TSK separation showed all components of the core oligosaccharide (Kdo, Gal, Hep) and of the O-specific chain (Man, GalNac) in both the GC-MS analysis and the NMR analysis and therefore was worked up further.

In this regard, we first checked whether analytical high-pressure anion-exchange chromatography (HPAEC) is suitable for purifying the oligosaccharides to homogeneity. To this end, we used a specific HPLC method for the analysis of complex sugar structures (DIONEX system) with an analytical CarboPac PA1 column (4.6 mm × 250 mm) and a linear salt gradient (5 min at 0 *M* NaOAc, then increased to 0.5 *M* NaOAc in 50 min) at a flow rate of 1 mL/min. The eluate was detected by a pulse-amperometric detector (PAD) for reduction equivalences (sugar molecules). Four oligosaccharide fractions were obtained in this way, which were then similarly further purified by semipreparative HPAEC.

The semipreparative HPAEC was carried out with a CarboPac PA1 column [(9 mm × 250 mm) Dionex system] with the same salt gradient as in the analytical HPAEC (5 min at 0 *M* NaOAc, then increased to 0.5 *M* NaOAc in 50 min) and a flow rate of 4 mL/min. The application of the oligosaccharide (42 mg; pool A from the TSK column) to the semipreparative HPAEC column was performed in two analogous HPAEC runs. The eluate was collected in fractions of one minute each, and the individual fractions were analyzed by analytical HPAEC. Two principal fractions were obtained in this way by semipreparative HPAEC (fraction I, retention time $t_R$ ~ 12 min and fraction II, $t_R$ ~ 15 min). The

salt had to be removed from both HPAEC fractions by means of a G-10 column (2.5 × 120 cm) before the MALDI-TOF-MS and NMR analysis (yield: fraction I: 4.68 mg; fraction II: 4.39 mg).

## MATRIX-ASSISTED LASER DESORPTION/IONIZATION TIME-OF-FLIGHT (MALDI-TOF) MASS SPECTROMETRY

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was recorded in a Bruker-Reflex$^{II}$ time-of-flight spectrometer (Bruker-Franzen Analytik, Bremen) exclusively in the linear configuration and in the negative mode at an accelerating voltage of 20 kV and with delayed ion extraction. The samples were first dissolved in chloroform (lipid A) or distilled water (oligosaccharide fractions) in a concentration of 10 μg/μL. 2-μL aliquots of these solutions were dissolved with 2 μL of a matrix solution consisting of 0.5 *M* 2,4,6-trihydroxyacetophenone (Aldrich, Steinheim) in methanol. Aliquots (0.5 μL) of this mixture were applied to a metal holder and dried with a hair drier.

## NMR SPECTROSCOPY

One-dimensional (1D) $^{1}H$- and $^{31}P$-NMR spectra and two-dimensional (2D) NMR spectra were recorded with a Bruker Avance DRX-600 spectrometer (Bruker, Rheinstetten), and $^{13}C$ NMR spectra were recorded with a Bruker AMX-360 spectrometer at 300 K in $^{2}H_2O$. Before each measurement, the samples were lyophilized twice with heavy water ($^{2}H_2O$). Acetone ($\delta_H$ 2.225 ppm, $\delta_C$ 31.45 ppm) or 85% $H_3PO_4$ ($\delta_P$ 0 ppm) was used as the external reference signal. Standard Bruker software (XWINNMR 1.3) was used to record the NMR data. The mixing times for the TOCSY (total correlated spectroscopy) and NOESY (nuclear Overhauser enhancement spectroscopy) were 100 and 500 ms, respectively.

## SEROLOGIC ANALYSES

The serologic analyses were performed as Western blots, which were developed with three different antibodies.

1. Polyclonal anti-O6 antiserum (rabbit) was prepared with *E. coli* strain DSM 6601 (serotype O6:K5:H1) at the Institute of Hygiene in Hamburg (Prof. Bockemühl).

2. Polyclonal anti-*E. coli* R1-antiserum (rabbit, internal designation: K299/d58) was obtained by immunization with a rough-form mutant that possesses an R1-core (anti-R1).

3. A monoclonal antibody (WN1-222-5, internal designation: F 167) was used, which broadly cross-reacts against all *E. coli* core oligosaccharides above a minimal structure (> Rd).

TABLE:

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Component analysis of E. coli LPS extracted from the strain DSM 6601.

| Component | Amount of the component nmoles/mg (moles/LPS)[a] | |
|---|---|---|
| *Carbohydrates* | Analysis 1 | Analysis 2 |
| GlcN[b] | 283 (1.8) | not determined |
| GalN | 139 (0.9) | not determined |
| HexN[c] | 591 (3.8) | 589 (2.9) |
| Kdo | 248 (1.6) | 242 (1.2) |
| Man | 321 (2.1) | 383 (1.9) |
| Gal | 474 (3.0) | 557 (2.8) |

| | | |
|---|---|---|
| Glc | 1069 (6.9) | 1291 (6.4) |
| L,D-Hep | 566 (3.6) | 442 (2.2) |
| *Polar Head Groups* | | |
| P | 1188 (7.6) | 1146 (5.7) |
| Etn-P | 85 (0.5) | not determined |
| *Fatty Acids* | | |
| 12:0 | 130 (0.8) | 162 (0.8) |
| 14:0 | 156 (1.0) | 201 (1.0) |
| 14:0(3-OH) | 460 (3.0) | 504 (2.5) |
| 16:0 | traces | traces |

[a] The molar ratio of the individual components (in parentheses) was standardized to the value of myristic acid (14:0) (1.0 mole 14:0/mole LPS)due to the presence of GalNac and GlcNac in the O-specific chain.

[b] GlcN determined by amino acid analyzer. The value is obtained from the sum of GlcN and GlcN-6P.

[c] HexN photometrically determined by the Morgan-Elson test.

The LPS preparations obtained by the method described above and comparative samples of LPS were subjected to polyacrylamide gel electrophoresis (cf. Figure 1). In the preparation of the SDS-PAGE analysis of the LPS, we worked with 16% polyacrylamide gels (U.K., Laemmli, Cleavage of Structural Proteins during Assembly of Head of Bacteriophage T4, Nature, 227, 680-685 (1970)). The LPS bands were stained by the sensitive alkaline silver

stain method (C. M. Tsai and Frasch, C. F., A Sensitive Silver Stain for Detecting Lipopolysaccharides in Polyacrylamide Gels, *Anal. Biochem.*, 119, 1982, 115-119).

The results of the analyses are shown in Figure 1.

## E X A M P L E 3

### BIOLOGICAL ACTIVITY

### (a) IL-1 activity

The IL-1 activity is determined by an MNC proliferation assay in a culture supernatant. Human monocytes (MNC) are isolated from the peripheral blood of volunteer donors ($8 \times 10^5$ MNC/200 mL), transferred into a glass and simultaneously treated with test substance. To test the biological activity *in vitro*, the cells are first stimulated with LPS (10 ng/mL). After an incubation period of 8 hours, 150 mL of the culture supernatant are analyzed for cytokine release. The IL-1 activity is determined by a fibroblast proliferation assay in a culture supernatant. The fibroblasts needed for this were obtained from human prepuce. The proliferation of these fibroblasts was increased by IL-1. The biological activity in the culture supernatant is determined by comparing the dose-response curve of the culture supernatant with the curve of the standard in a probit analysis. The LPS from a bacterial strain known to be endotoxically active (*Salmonella friedenau*) serves as the reference (positive control) and therefore is included in Figure 5.

### (b) TNFα activity

The TNFα activity in a culture supernatant is determined in a cytotoxicity assay with the TNF-sensitive cell line L929. The TNF activity can be determined by comparing the dos-response curve of the culture

supernatant with the curve of the standard in a probit analysis. Here again, a *Salmonella friedenau* LPS that is known to be endotoxically active serves as the positive control. The results are graphically represented in Figure 6.

The results show that, with respect to IL-1 and TNFα release, there are no significant differences between the LPS from *Salmonella friedenau*, which serves as the standard and positive control, and the LPS from the strain DSM 6601 (Figures 5 and 6, lower graphs). This is also confirmed by the fact that the lipid A of the strain DSM 6601 shows virtually the same activity as the highly purified lipid A of E. coli (Figures 5 and 6, upper graphs).

CLAIM(S)

1. Lipopolysaccharide (LPS) with the structure shown in Figure 7.

2. LPS in accordance with Claim 1, characterized by a content of 8 phosphate groups per molecule of LPS.

3. LPS in accordance with Claim 1 or 2, characterized by a content of 0.5 mole of P-Etn per mole of LPS.

4. Process for producing LPS in accordance with Claims 1 to 3, characterized by the fact that a washed and dried *E. coli* bacterial mass is subjected to a phenol/water extraction, which is already well known in itself, and the resulting extract is subjected to a treatment with RNases/DNases and proteinase K.

5. Process in accordance with Claim 4, characterized by the fact that *E. coli* strain DSM 6601 is used.

6. Use of lipopolysaccharide from *E. coli* strain DSM 6601 in accordance with Claims 1 to 3 for microbiological, bioengineering, analytical, diagnostic and/or medical purposes.