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Note

A very efficient method to cleave Lipid A and saccharide components in bacterial lipopolysaccharides

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Abstract

A novel mild procedure for the selective cleavage of ketosidic linkages is developed using ceric ammonium nitrate (CAN) in anhydrous *N*,*N*-dimethylformamide. Its application to lipopolysaccharides (LPS) is very significant because in the so far investigated LPS, the connection between the Lipid A region and the oligo(poly)saccharide part is always a keto-sugar. This procedure has been tested on LPS of *Escherichia coli* which contains Kdo as a linker between Lipid A and OPS and on *Acinetobacter haemoliticus* which contains D-*glycero*-D-*talo*-2-octulopyranosonic acid (Ko) as a linker and it performed efficiently in both cases.

Keywords: Ceric ammonium nitrate; Ketosidic cleavage; Kdo; D-*glycero*-D-*talo*-2-Octulopyranosonic acid; Gram-negative bacteria; O-Antigen; Endotoxin

Lipopolysaccharides (LPS, endotoxins) are amphiphilic molecules which are present in the outer membrane of most Gram-negative bacteria. They are constituted of a carbohydrate moiety of varying size, consisting in most cases of a polysaccharide composed of repeating units (O-chain, O-specific polysaccharide, O-antigen) that is linked to an oligosaccharide (the core region), which in turn substitutes a lipid moiety (Lipid A). The LPS exhibit antigenic properties, and can act as potent virulence factors and are well known as bacterial endotoxins, which play an important role in the pathophysiology of Gram-negative sepsis.[1]

One of the classical strategies in structural analysis of LPS involves the cleavage of the ketosidic linkage of 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo), by which the core region is bound to the Lipid A. This particular cleavage must occur to preserve the other glycosidic bonds in order to allow the structural study of a non-degraded polysaccharide moiety. Thus, exploiting the acid lability of the ketosidically linked Kdo residue, mild-acid conditions can be used, e.g., hydrolysis in 1% aqueous acetic acid at 100 °C between 30 min and 3 h. Aldosidic linkages are preserved under such conditions, except those of other acid-labile monosaccharides, such as 2-deoxy and 3-deoxy sugars.[2] On the other hand, bacteria of the genus *Acinetobacter* may replace Kdo in their LPS by the more acid resistant D-*glycero*-D-*talo*-2-octolusonic acid (Ko), therefore more drastic acid conditions are required to isolate the O-specific polysaccharide.[3] Taken together, the search for novel and milder conditions to accomplish the separa-

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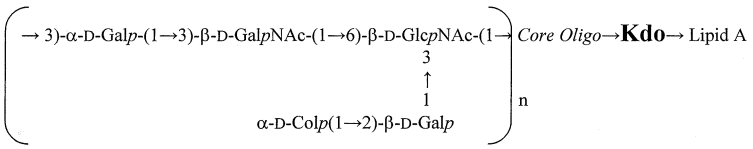

Fig. 1. The structure of the O-specific polysaccharide from LPS of *E. coli* O55:B5.

tion of Lipid A and the saccharide part is still an important goal.

We have developed a novel procedure for the cleavage of ketosidic linkages using ceric ammonium nitrate (CAN) in anhydrous *N*,*N*-dimethylformamide (DMF), e.g., sucrose is cleaved at room temperature after 24 h, while aldobioses (maltose, cellobiose and gentiobiose) are not cleaved at all. An important application of this methodology is the cleavage of the ketosidic linkage in LPS.

The LPS of *Escherichia coli* O55:B5[4] (Fig. 1) and CAN (1:3, w/w) were dissolved in anhydrous DMF and kept at 20–22 °C. The progress of the reaction was followed by sodium dodecylsulfate (SDS) polyacrylamide gel-electrophoresis (PAGE) on samples taken at different reaction times. After 4 h reaction time, LPS could not be detected in the gel, thus indicating a quantitative cleavage of LPS.

When the reactions of LPS with 1% AcOH and CAN were compared, the same spectrum and quantities of fatty acids were detected by GLC–MS analysis of the Lipid A fractions. However, no phosphate could be detected by ^{31}P NMR spectroscopy and MALDI-TOF mass spectrometry in Lipid A obtained from the reaction with CAN, which is in agreement with its dephosphorylating properties.[5] With regard to that, cleavage and separation of LPS into Lipid A and polysaccharide fractions utilizing CAN is not suitable for phosphate determination. On the other hand it can be considered as a one-step procedure to cleave and dephosphorylate Lipid A.

Table 1
Compositional analysis of the O-specific polysaccharides isolated from LPS of *E. coli* O55:B5

	Gal	GalNAc	GlcNAc	Col
1% AcOH	2	1	1	0.5
CAN/DMF	2	1	1	0.9

The efficiency of this method in case D-*glycero*-D-*talo*-2-octulopyranosonic acid (Ko) is replacing Kdo was tested on a sample of de-*O*-acylated LPS from *Acinetobacter haemolyticus* strain NCTC 10305, in which Ko replaces Kdo at about 80%.[6] The reaction, which was performed under the same conditions used for LPS of *E. coli*, was completed after 4 h as indicated by SDS–PAGE. It is worth noting that by using acid hydrolysis, the cleavage of the ketosidically linked Ko occurred only under rather strong conditions, which cleaved other glycosidic linkages. Thus, the herein described method represents a valid and milder way to obtain without any side reaction with the O-specific polysaccharide of LPS in which D-*glycero*-D-*talo*-2-octulopyranosonic acid replaces Kdo.

A comparison of the data obtained from compositional analyses of the O-specific polysaccharides isolated from LPS of *E. coli* O55:B5 after cleavage with AcOH and CAN revealed that in the latter case, the acid-labile colitose was cleaved in a low percentage only (less than 10%), while with AcOH it is cleaved in a higher percentage (50%, Table 1). It is known that hydrolysis in AcOH cleaves, not only Kdo, but also few other deoxy-sugars like caryophyllose or colitose,[4,7] while the CAN reaction seems to be specific for 2-ulosonic acids, i.e., Kdo and Ko. A possible explanation is that Kdo and Ko react faster because a coordination between their carboxyl group and the Ce^{IV} ion keeps the first close to the interglycosidic oxygen.[8]

Since *O*-acetyl groups are frequent decorations of O-specific polysaccharides, we have investigated also their stability in the reaction with CAN. A test was carried out using 2,3-di-*O*-acetyl-α-D-*O*-methylglucopyranoside and acetylated polysaccharide (acetyl pullulan). After treatment with CAN in anhydrous

DMF for 4 h, they were recovered unaltered as observed by NMR data. Hence, in agreement with the stability of the fatty acids in Lipid A, also *O*-acetyl groups substituting monosaccharides are not cleaved by this method.

In conclusion, treatment with CAN/anhydrous DMF appears to be a mild and efficient method to cleave the ketosidic linkage between Lipid A and O-specific polysaccharide in LPS. Since it is a ketosidic linkage that connects these two regions of LPS, the reaction with CAN is the only method, so far, by which this linkage can be cleaved, independent of the ketose that is present.

1. Experimental

General methods.—The LPS from *E. coli* O55:B5 was purchased from Sigma–Aldrich whereas the de-*O*-acylated LPS from *A. haemolyticus* was a kind gift of Professor O. Holst (Forschungszentrum Borstel, Germany). ^{31}P NMR spectra were recorded in the FT mode with a Bruker DRX 400 spectrometer equipped with a reverse probe head, at 30 °C. The samples were dissolved in DMSO-d_6, and 85% phosphoric acid was used as an external standard. SDS/PAGE (12 or 18% acrylamide) and staining with silver nitrate were performed as described.[9] Sugar analysis was performed by GLC–MS of acetylated *O*-methyl glycosides. Briefly, samples (LPS, supernatants and precipitates) were methanolyzed with 1 M methanolic HCl at 80 °C for 18 h, dried in a stream of N_2 and then acetylated with Ac_2O in pyridine at 20–22 °C for 18 h. After work-up, the sample was analyzed by GLC–MS with a Hewlett–Packard 5890 gas chromatograph equipped with a SPB-5 capillary column (0.25 mm × 30 m, Supelco), applying the temperature program 150 °C for 5 min, then 3 °C/min to 330 °C. Fatty acids were obtained after methanolysis of the LPS and extraction of the sample with *n*-hexane. Their analysis was performed by GLC–MS with the temperature program 150 °C for 3 min, then 10 °C/min to 280 °C over 20 min. Alternatively, fatty acids were analyzed after successive hydrolyses in 4 M HCl at 100 °C for 4 h and 4 M NaOH at 100 °C for 30 min. Then the pH was adjusted to slight acidity, and the fatty acids were extracted with $CHCl_3$ and esterified with diazomethane. Finally, they were analyzed by GLC–MS as described above.

Cleavage of sucrose.—Sucrose was treated with CAN in anhyd DMF at 20–22 °C. After 24 h, the reaction products were identified as acetyl derivatives by GLC–MS as glucose and fructose(traces) and its 2,6-anhydrous derivative. Aldobioses are not cleaved in the above conditions.

Hydrolysis of LPS from E. coli *O55:B5.*—The sample was dissolved in 1% aq AcOH and hydrolyzed at 100 °C for 2 h. Then, the sample was centrifuged (12,000*g*) yielding the O-specific polysaccharide (supernatant) and the Lipid A (precipitate).

Cleavage of LPS from E. coli *O55:B5 with CAN.*—LPS (10 mg) and 32 mg of CAN were dissolved in 1.5 mL of anhyd DMF. The reaction mixture was stirred at 20–22 °C for 4 h, and was then quenched with 1 mL of pyridine. Afterwards, 15 mL of NH_4HCO_3 (0.25 M) were added and the mixture was lyophilized. The dry sample was suspended in water containing a drop of pyridine and centrifuged (4 °C, 12,000*g*, 60 min.), yielding a supernatant (the O-specific polysaccharide, 42 mg) and a precipitate (the Lipid A, 4 mg). The O-specific polysaccharide was purified by gel-permeation chromatography (TSK-40 in water; column size 1 × 44 cm; flow: 20 mL/h; fraction volume: 1 mL), using a differential refractometer (Knauer, Germany) for monitoring (yield: 8 mg, 80% of the LPS). The Lipid A was recovered as reported.[10] Briefly, after TLC on silica gel 60 developed in (40:60:16:5 v/v) $CHCl_3$–MeOH–Py–85% formic acid, a single band with R_f 0.7 was scraped out, and the silica gel was extracted with 20 mL of (1:2:0.8 v/v) $CHCl_3$–MeOH–50 mM (pH 1.5) aq ammonium acetate for 40 min, centrifuged (2500*g*, 5 min), and the supernatant was then filtered. Then, 6 mL of $CHCl_3$ and 6 mL of 50 mM (pH 1.5) aq ammonium acetate were added to the filtered sample, and the lower phase was recovered, neutralized with pyridine and evaporated.

Cleavage of Ko in LPS from A. haemolyticus *strain NCTC 10305 with CAN.*—LPS (4 mg) and 13 mg of CAN were dissolved in 0.6 mL of dry DMF and the reaction was stirred at 20–22 °C for 4 h. The reaction was quenched with 0.5 mL of pyridine, then 8 mL of NH_4HCO_3 (0.25 M) were added and the mixture was lyophilized. The dry sample was suspended in water containing a drop of pyridine and centrifuged (4 °C, 12,000*g*, 60 min.), obtaining a supernatant (12 mg) and a precipitate (4 mg). The supernatant was chromatographed by gel-permeation chromatography (TSK-40 in water; column size 1 × 44 cm; flow: 20 mL/h; fraction volume: 1 mL), using a differential refractometer (Knauer, Germany) for monitoring. Two carbohydrate-containing fractions and a salt fraction were obtained.

*2,3-Di-*O*-acetyl-α-*D*-O-methylglucopyranoside.*—The compound was obtained by acetylation of 4,6-benzylidene-α-D-*O*-methylglucopyranoside (Sigma) with Ac_2O in pyridine at 20–22 °C for 18 h. After working up, the product was dissolved in (2:1:10 v/v) trifluoroacetic acid–water–$CHCl_3$ and stirred at 20–22 °C for 30 min. Finally, the compound was purified by TLC developed in (9:1 v/v) $CHCl_3$–MeOH.

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