methods

Ammonium hydroxide hydrolysis: a valuable support in the MALDI-TOF mass spectrometry analysis of Lipid A fatty acid distribution

Alba Silipo, Rosa Lanzetta, Angela Amoresano, Michelangelo Parrilli, and Antonio Molinaro[1]

Dipartimento di Chimica Organica e Biochimica, Università degli Studi di Napoli Federico II, Via Cintia 4, I-80126, Napoli, Italy

Abstract **Lipid A is the lipophilic moiety of lipopolysaccharides (LPSs), the major components of the external membrane of almost all gram-negative bacteria. It is responsible for the toxicity of LPS and has a heterogeneous structure composed of a bis-phosphorylated glucosamine disaccharide backbone that is acylated at the positions 2, 3 of the GlcN I (proximal) and GlcN II (distal) residue with *O*- and *N*-linked 3-hydroxy fatty acids (primary substitution). These fatty acids are further acylated by means of their 3-hydroxy groups (secondary substitution). The toxicity of Lipid A is dependent on its primary structure; the number, the length, and the distribution of the fatty acids on the disaccharide backbone strongly influence the endotoxic activity. In this paper a general and easy methodology to obtain secondary fatty acid distribution, which is one of the most difficult issues in the structural determination of Lipid A, is proposed. The method combines ammonium hydroxide hydrolysis and matrix assisted laser desorption ionization (MALDI)-mass spectrometry analysis and has been successfully proven with five different Lipid A species. The procedure exploits the lower stability under mild alkaline conditions of acyl and acyloxyacyl esters with respect to that of the acyl and acyloxyacyl amides. The partially degraded Lipid A species obtained are analyzed by MALDI-MS. The generality of this approach was tested on five Lipid As, namely those arising from *Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Pseudomonas reactans*, and *Burkholderia caryophylli*.**—Silipo, A., R. Lanzetta, A. Amoresano, M. Parrilli, and A. Molinaro. **Ammonium hydroxide hydrolysis: a valuable support in the MALDI-TOF mass spectrometry analysis of Lipid A fatty acid distribution.** ***J. Lipid Res.*** **2002.** 43: **2188–2195.**

Supplementary key words ammonium hydroxide • endotoxin • gram-negative bacteria

Lipopolysaccharides (LPSs) are the components of the external membrane of almost all gram-negative bacteria. They usually constitute a hydrophilic moiety consisting of the *O*-specific chain (*O*-antigen) and the core oligosaccharide, covalently linked to a lipophilic moiety (Lipid A) that anchors LPS to the outer membrane. Lipid A constitutes the endotoxic principle of the LPS molecule, expressing all the pathophysiological effects known to be induced by these molecules.

From a structural point of view, Lipid A usually consists of a β-1,6-D-glucosammine backbone bisphosphorylated at C-1 and C-4′ positions, acylated with β-hydroxylated fatty acids (primary substitution), linked as ester at C3 and C3′ positions and as amides at C2 and C2′ positions. Non-hydroxylated or, less frequently, α-hydroxy fatty acids (secondary substitution) can be connected in ester linkage at C-3 position of primary fatty acids (**Fig. 1**) (1, 2). Lipid A is usually characterized by a micro-heterogeneity mainly due to the type, number, and position of the fatty acids; this heterogeneity can be further increased by the variability of phosphate substitution, e.g., by additional sugar or other substituents linked to the phosphate groups.

The fatty acid distribution, their length, and the site of attachment strongly influence the toxicity properties of this molecule.

Lipid A is obtained by mild acid hydrolysis from LPS, and the classical approach to its structural characterization begins with the compositional analysis, specifically phosphate, fatty acids, and monosaccharide content. The next steps consist in the determination of: *a*) the linkage between the glucosamine units (to date, exclusively 1→6), *b*) the ester-linked fatty acids, and *c*) the amide-linked fatty acids.

In this regard, the following degradation methods can be used: *a*) methylation analysis, and *b*) acid and alkaline hydrolysis followed by methylation and GC-MS analysis of the methyl ester derivatives of the *O*-acyl and *N*-acyl fatty acids (3).

One- and two-dimensional nuclear magnetic reso-

Manuscript received 25 May 2002 and in revised form 5 August 2002.

Published, JLR Papers in Press, September 16, 2002.
DOI 10.1194/jlr.D200021-JLR200

[1] To whom correspondence should be addressed.
e-mail: molinaro@unina.it

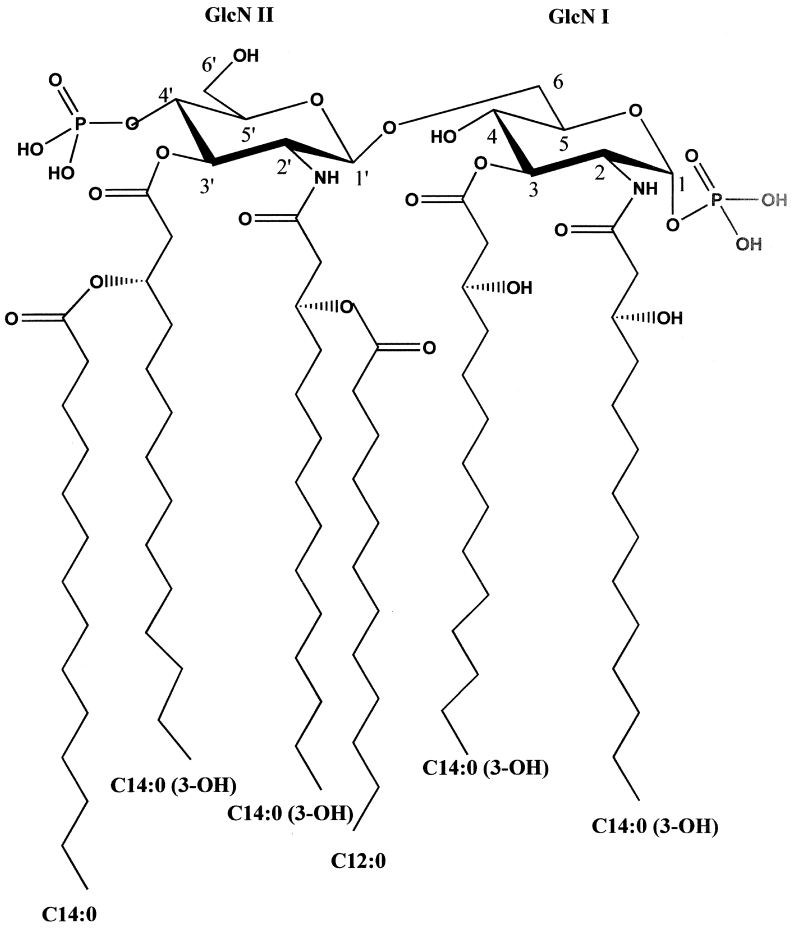


Fig. 1. The Lipid A structure of *Escherichia coli* showing the disaccharide backbone and primary and secondary fatty acids.

nance of ^{1}H, ^{13}C, and ^{31}P are used to establish the sugar backbone (anomeric configurations) and the location of phosphate groups (4–6). Several mass spectrometry techniques are exploited to gain information about the heterogeneity, i.e., the number of different species of the Lipid A families and distribution of the fatty acids on each glucosammine unit (7–12). The identification of the oxonium ion peaks arising from the cleavage of glycoside bond can be very useful in obtaining information on the GlcN II substituents. However, all these chemical and spectroscopic methods could hardly allow us to identify the position of the secondary fatty acids (3), i.e., whether they are present as acyloxyacyl amide or acyloxyacyl ester on each GlcN residue.

To this purpose, structural information can be achieved by matrix assisted laser desorption ionization-time of flight (MALDI-TOF) MS analysis of the partially degraded Lipid A obtained by mild hydrolysis with ammonium hydroxide. This procedure is able to split selectively the acyl and acyloxyacyl esters, leaving the acyl and acyloxyacyl amides unaffected. Previous studies have already tested the utility of the mild hydrolysis to obtain a selective de-*O*-acylation even if no systematic study of mild alkaline conditions has been carried out so far (3, 13–16).

EXPERIMENTAL PROCEDURES

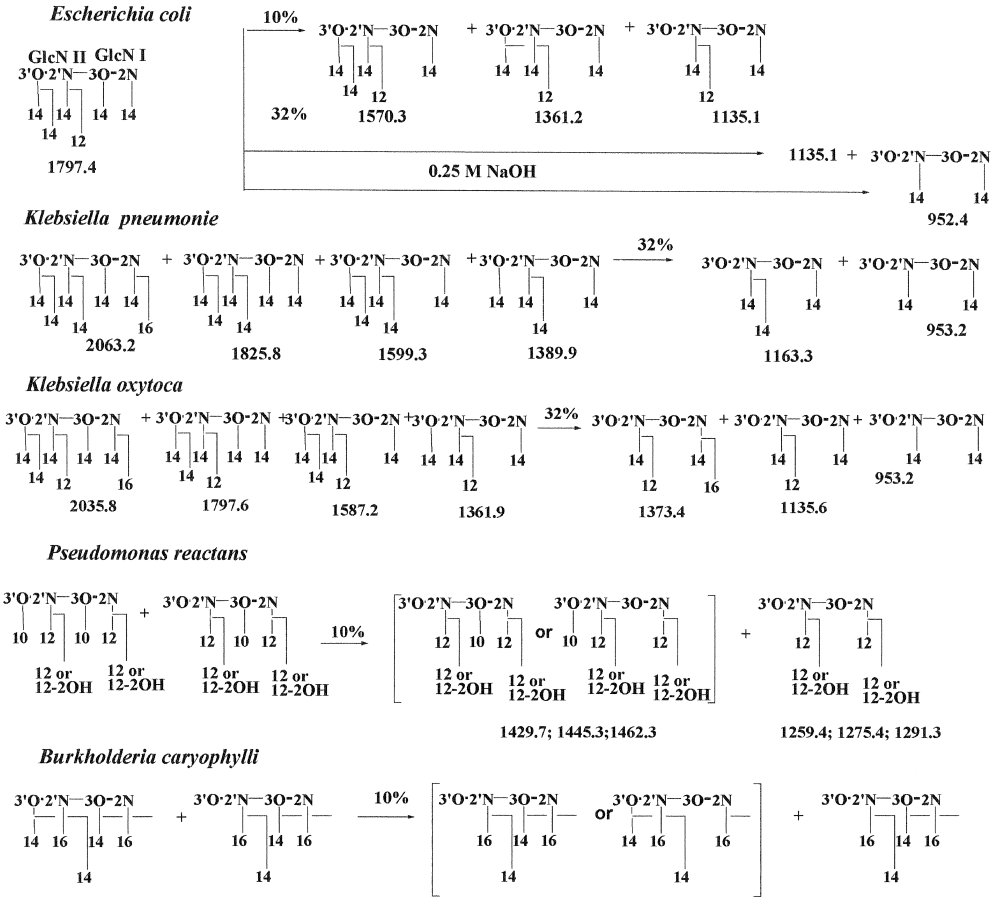
Growth of bacteria, isolation of LPSs and Lipid A

LPSs from *Pseudomonas reactans* strain NCPPB 1311 and *Burkholderia caryophylli* strain NCPP 2151 were obtained as described in references (20–21) with hot phenol-water method (22). LPS of *Escherichia coli* strain 055:B5 was purchased from Sigma and LPSs from *Klebsiella oxytoca* (19). Strain R29, and *Klebsiella pneumoniae* (18) strain R20 were a gift from Dr. O. Holst (Research Center Borstel).

In order to obtain the Lipid A, LPSs were hydrolyzed with 0.1 M sodium acetate buffer (15 mg/ml) at pH 4.4, containing 0.1% SDS at 100°C for 2 h. Then the solution was lyophilized, extracted once in 2M HCl/ethanol and twice with ethanol, dried, re-dissolved in water, and ultracentrifuged (110,000 *g*, 4°C, 1h). The sediment was washed four times with water and lyophilized (Lipid A, yield: 6% of LPS).

De-*O*-acylation of Lipid A with ammonium hydroxide solutions

Lipid A (0.2 mg) from *E. coli*, *P. reactans*, and *B. caryophylli* was treated with a 1:3 diluted NH_4OH solution (200 μl) for 16 h at room temperature; in the same condition (200 μl, room temperature, 16 h), Lipid A from *E. coli*, *K. pneumoniae*, and *K. oxytoca* was treated with conc. NH_4OH (200 μl) solution. The samples were simply dried under nitrogen and directly analyzed by mass spectrometry (17).



Scheme 1. Sketches of Lipid A structures corresponding to the ions occurring in the MALDI-TOF MS spectra showed along the paper. All the structures must be intended as phosphorylated.

De-*O*-acylation of Lipid A of *E. coli* with 0.25 M sodium hydroxide solution

Lipid A (0.2 mg) from *E. coli* was treated with 0.25 M NaOH for 15 min (14). The product was neutralyzed, desalted on a filter, and analyzed by mass spectrometry.

MALDI-TOF-MS analysis

Negative and positive MALDI-TOF mass spectra were recorded using a reflectron Voyager DE-PRO MALDI-TOF mass spectrometer (Applied Biosystem): 1 μl analyte solution in chloroform-trifluoroethanol (4:1, v/v) was mixed to 1 μl of matrix solution consisting of 2.5 dihydroxy benzoic acid in acetonitrile-0.2% TFA, (70:30, v/v), applied to the sample plate, and dried down. Mass calibration was performed using an external calibration. Raw data were analyzed by using computer software provided by the manufacturer and are reported as average masses.

RESULTS AND DISCUSSION

E. coli Lipid A analysis

Fatty acids analysis was in full agreement with the known structure (2, 17) and revealed the presence of two C14:0 (3-OH) (as primary fatty acids), one C14:0, and one C12:0 residue as in ester linkage, and two primary C14:0 (3-OH) in amide linkage. The negative MALDI-MS spectrum showed a quasimolecular ion peak $(M\text{-}H)^-$ at *m/z* 1797.4 corresponding to a hexaacyl residue (**Scheme 1**). The identification of the oxonium ion in the positive MALDI-MS allowed the location of two C14:0 (3-OH), one C14:0, and one C12:0 residues on the GlcN II; as a consequence, two primary C14:0 (3-OH) residues are present on the GlcN I.

From these data it is not possible to assign the exact location of C14:0 and C12:0 secondary fatty acids, e.g., which is on the *N*-acyl and which is on the *O*-acyl primary fatty acid.

The observation that with 0.25 M NaOH at 37°C for 15 min (14) the acyloxyacyl ester linkage is cleaved whereas the acyloxyacyl amide is unaffected prompted us to see if this is a general fashion and if it is possible to increase the selectivity of this hydrolysis. To this aim, we have exploited the ammonium hydroxide that is a weaker base than NaOH and in addition has the advantage to be completely and easily removed by only drying the sample under vacuum.

We have experimented with both conditions (NaOH 0.25M and NH_4OH sol. conc.) and a 1:3 dilute NH_4OH solution at room temperature for 16 h. When the Lipid A of *E. coli* was allowed to react with dilute NH_4OH solution, the resulting negative MALDI-TOF mass spectrum (**Fig. 2A**) showed ion peaks that could be identified (see Scheme 1) as follows: the ion at *m/z* 1571.3 is univocally explained

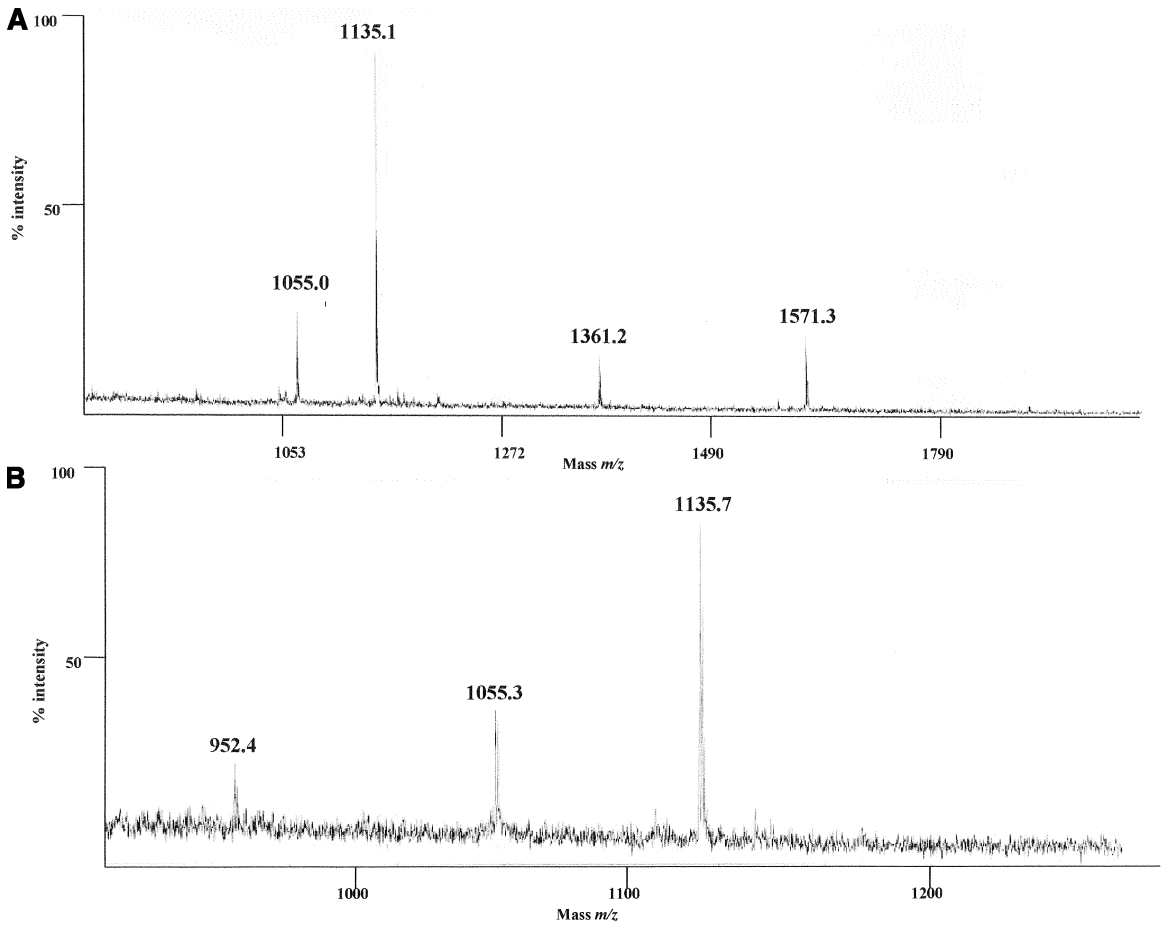


Fig. 2. Negative ion MALDI-MS spectra of the Lipid A of *E. coli* obtained from hydrolysis with (A) dilute NH_4OH and (B) conc. NH_4OH.

with the absence of a C14:0 (3-OH) residue obviously lost from GlcN I; the ion at *m/z* 1361.2 accounts for the additional lack of the secondary fatty acid C14:0 from the ester linked primary fatty acid at 3′ position; the ions at *m/z* 1135.1 accounts for the absence, from the species at *m/z* 1361.2, of the primary C14: 0 (3-OH) at 3′ position. The peak at *m/z* 1055.0 is the corresponding monophosphorylated species. The ion at *m/z* 1135.1 can only be explained with the presence of a secondary fatty acid C12:0 on the primary C14: 0 (3-OH) *N*-linked to GlcN II, thus allowing the definitive assignment of the C12:0 location.

In the spectrum of the product obtained using the conc. NH_4OH solution (Fig. 2B), only ions at *m/z* 1135.7 and at *m/z* 1055.3 were present, deriving from the total and selective hydrolysis of acyl esters and acyloxyacyl esters, respectively, while at *m/z* 952.4 a minor ion was present that corresponded to diacyl species lacking the secondary C12:0 residue at position 2′ (Scheme 1). This last peak was the only ion present in the MALDI-TOF spectrum (data not shown) when the 0.25 M NaOH aqueous solution was used under the described conditions (14).

The two MALDI-TOF spectra of the product obtained with both NH_4OH concentrations allowed the punctual location of the secondary fatty acid: the C14:0 at the β position of the *O*-linked primary fatty acid and the C12:0 at the β position of the *N*-linked primary fatty acid. In particular, the dilute NH_4OH solution hydrolysis gave a higher selectivity and thus a higher efficacy in setting up the distribution of fatty acids: it permits the stepwise hydrolysis of acyl esters and acyloxyacyl esters, which can be followed on the MALDI-MS profile. Therefore, the analysis of all the ions in the negative MALDI-MS spectrum of the products of the NH_4OH cleavage, combined with the MALDI-MS analysis of native Lipid A, allowed us to define the complete fatty acid distribution.

To show the generality of this approach, this procedure was also tested on Lipid A arising from *K. pneumoniae*, *K. oxytoca*, *P. reactans*, and *B. caryophylli*.

K. pneumoniae Lipid A analysis

In the case of *K. pneumoniae,* the MALDI-TOF analysis was consistent with the literature data (18). The Lipid A fraction mainly consisted of hexaacyl species at *m/z* 1825.8 containing four C14:0 (3-OH) and two C14:0 residue. Minor fractions were attributed to pentacyl species at *m/z* 1599.3 lacking a C14:0 (3-OH) and to tetracyl species at *m/z* 1389.9 lacking a C14:0 (Scheme 1); traces of heptacyl species at *m/z* 2063.2, containing a C16:0 linked to the amide primary C14:0 (3-OH) of the GlcN I, were also found. Both secondary C14:0 fatty acids were located on the GlcN II residue.

Lipid A was hydrolyzed with conc. NH_4OH solution,

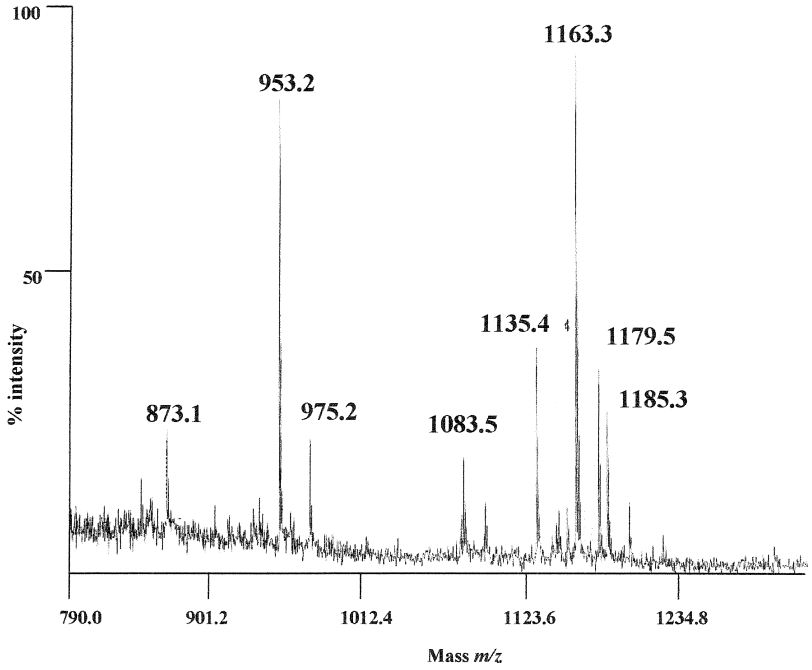


Fig. 3. Negative ion MALDI-MS spectrum of the Lipid A of *Klebsiella pneumoniae* obtained from conc. NH_4OH reaction. The lower peaks are sodium adducts or are originating from minor species of Lipid A of *K. pneumoniae*.

and the product was analyzed by MALDI-MS (**Fig. 3**). The spectrum showed a major ion at *m/z* 1163.3 corresponding to the triacyl species containing two C14:0 (3-OH) primary *N*-linked residues and a secondary residue C14:0 at 2′ position (Scheme 1). These results showed the expected ions in accordance with the ammonium hydroxide treatment; that is to say, one of the two acyloxyacyl residues is attached by amide linkage and is left mostly untouched with this reaction. A peak at *m/z* 953.2, corresponding to diacyl species and containing only the primary *N*-linked fatty acids and the corresponding monophosphoryl species at *m/z* 873.1, were also found.

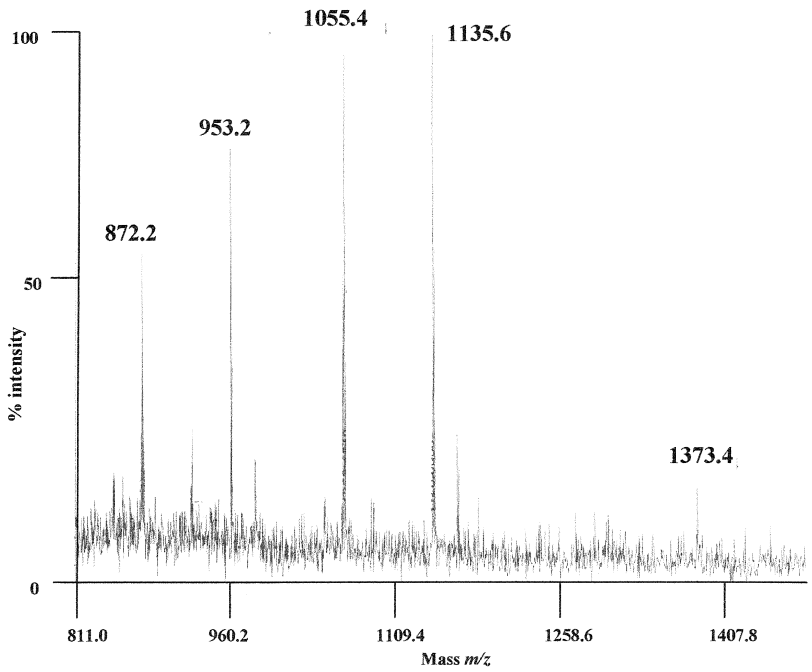


Fig. 4. Negative ion MALDI-MS spectrum of the Lipid A of *Klebsiella oxytoca* obtained from conc. NH_4OH reaction. The peak at *m/z* 1373.4 raises from the heptacyl species, whereas the others raise from the hexacyl species.

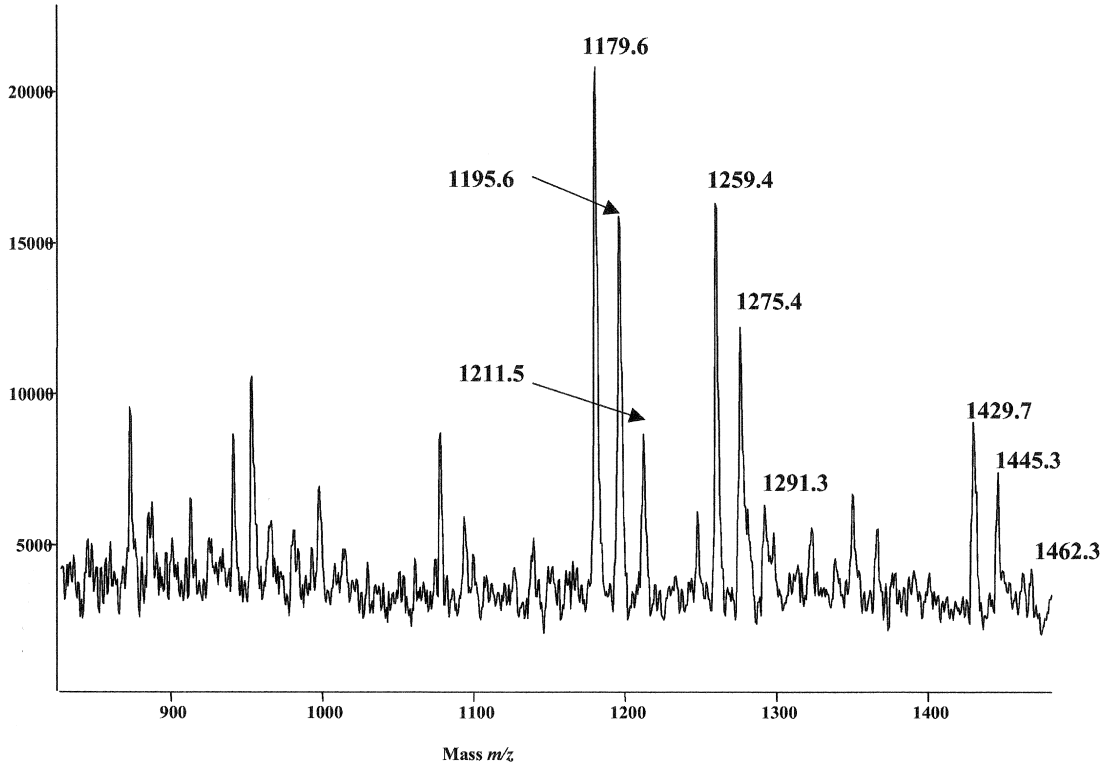


Fig. 5. Negative ion MALDI-MS spectrum of the Lipid A of *Pseudomonas reactans* obtained from 1:3 dilute NH_4OH solution reaction.

K. oxytoca Lipid A analysis

The MALDI-TOF MS spectrum of Lipid A from *K. oxytoca* was reliable with the literature data (19). It consists of a mixture of hexacyl species (*m/z* 1797.6) containing four C14:0 (3-OH), one C14:0, and one C12:0 residues, pentacyl species (*m/z* 1587.2) lacking the C14:0, and tetracyl species (*m/z* 1361.9) lacking a C14:0 (3-OH). Both secondary fatty acids C12:0 and C14:0 are located on GlcN II. Traces of heptacyl species at *m/z* 2035.8 with an additional secondary C16:0 on GlcN I were also found. The treatment of Lipid A fraction with the conc. NH_4OH solution gave the expected products. Thus, the MALDI-MS spectrum showed a minor peak at *m/z* 1373.4 diagnostic of a tetracyl species (**Fig. 4** and Scheme 1) arising from minor heptacyl species. It consisted of two acyloxyacyl amides in which the secondary fatty acids are C12:0 on the distal glucosamine and C16:0 on the proximal glucosamine, respectively. This was definitely proven by the presence of the oxonium ion peak found in the positive MALDI-MS spectrum (data not shown) generated from the cleavage of the glucoside linkage that can be retained a reliable proof of the structure.

Besides the peak at *m/z* 1373.4, arising from the minor heptacyl species, predictable peaks at *m/z* 1135.6 and 1055.4 (lacking a phosphate residue, $\Delta m/z = 80$), deriving from the major hexaacyl species, were found. These peaks were attributed to triacyl species bearing acyloxyacyl amides on GlcN II and an amide linked fatty acid C14:0 (3-OH) on GlcN I. Minor peaks at *m/z* 953.2 and 872.2 (monophosphoryl species) were attributed to a diacyl species carrying two *N*-linked C14:0 (3-OH).

P. reactans Lipid A analysis

The Lipid A fraction of *P. reactans* is rather complex and heterogeneous and contains a hexaacyl species (Scheme 1) with two C12:0 (3-OH) in amide linkage and two C10:0 (3-OH) in ester linkage; the secondary fatty acids, C12:0 (2-OH) or C12:0, are linked to the primary C12:0 (3-OH) amides. The pentacyl species is lacking the C10:0 (3-OH) at position C-3′ of distal glucosamine (20). The mild alkaline reaction with ammonium hydroxide was, in this case, really significant and helpful.

After the treatment with dilute NH_4OH solution, the Lipid A fraction was analyzed by MS (**Fig. 5** and Scheme 1). The MALDI-MS spectrum showed three ions at *m/z* 1429.7, 1445.3, 1462.3, and the corresponding monophosphoryl species at *m/z* 1349.7, 1365.7, 1381.7, diagnostic of pentacyl species lacking one of the two primary ester-linked C10:0 (3-OH) fatty acids. Further diagnostic peaks were present at *m/z* 1259.4, 1275.4, 1291.3, and at 1179.6, 1195.6, and 1211.5 ($\Delta m/z = 80$), indicative of tetracyl species with two acyloxyacyl amides in which the secondary fatty acids are C12:0 (2-OH) or C12:0. Thus, the selective hydrolysis of the two primary fatty acids ester linked allowed the univocal assignment of the secondary fatty acids to the β-position of the *N*-linked fatty acids, i.e., to the C12:0 (3-OH).

B. caryophylli Lipid A analysis

Only the fatty acid composition of the Lipid A from *B. caryophylli* was previously ascertained (2), and work is in progress to determine the whole structure. Two *O*-linked C14:0 (3-OH), two *N*-linked C16:0 (3-OH) as primary fatty acids, and one C14:0 as secondary residue were found in

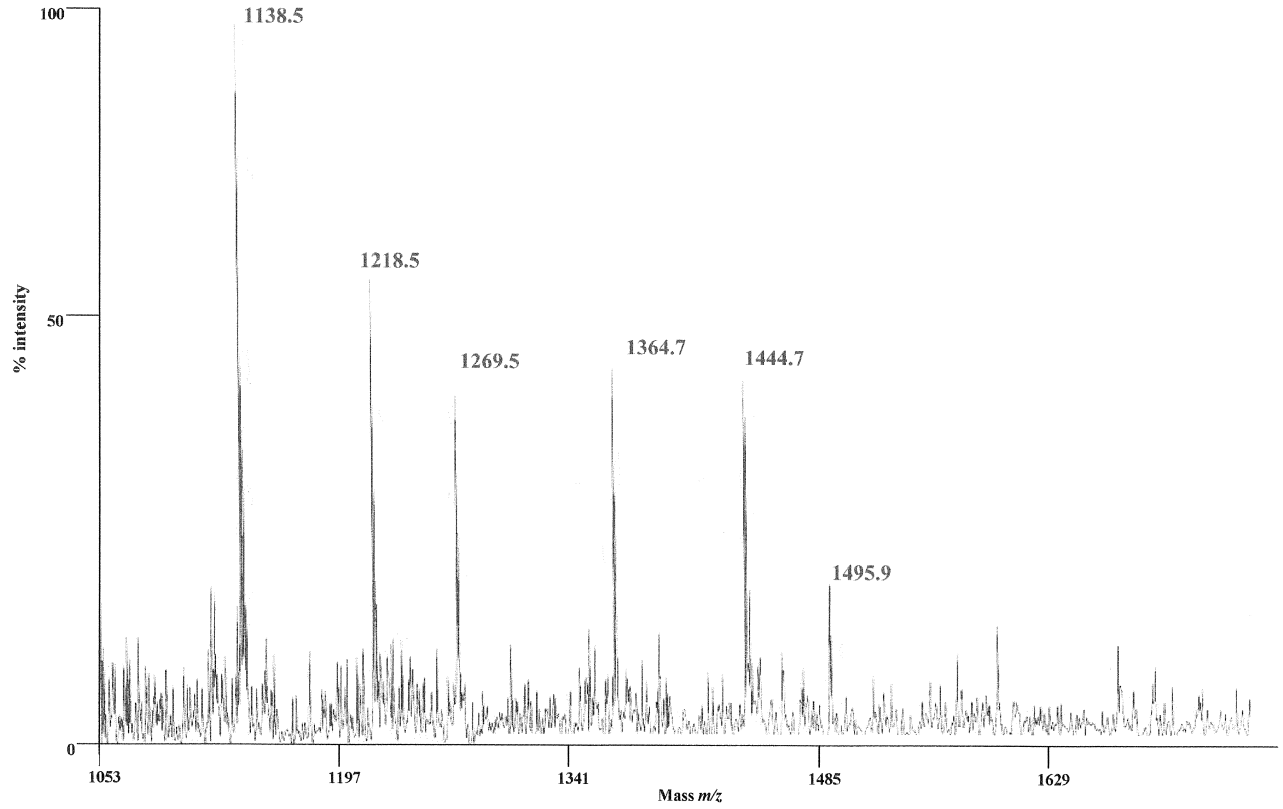


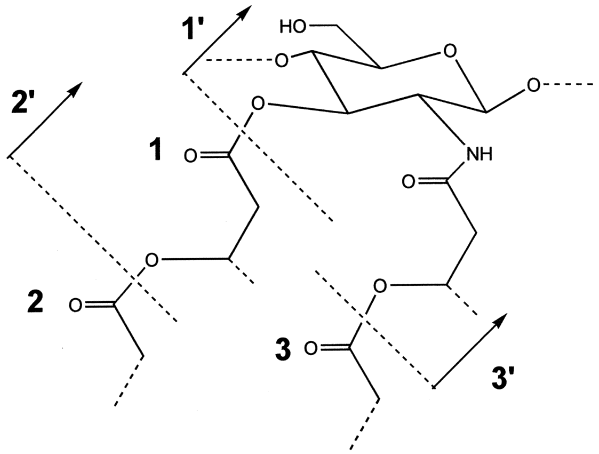
Fig. 6. Negative ion MALDI-MS spectrum of the Lipid A of *Burkholderia caryophylli* obtained from 1:3 dilute NH_4OH solution reaction. The ions not given are relative to the presence of an additional monosaccharide in the sugar backbone, the identification of which is now in progress.

the Lipid A fraction (Scheme 1). The location of C14:0 was unknown and was definitely placed on GlcN II as acyloxyacyl amide by using the ammonium hydroxide hydrolysis method with dilute NH_4OH solution. Actually, the MALDI-TOF MS spectrum revealed ions corresponding to triacyl species at *m/z* 1138.5 and 1218.5, and ions corresponding to tetracyl species at *m/z* 1364.7 and 1444.7 (**Fig. 6**). The ion at *m/z* 1218.5 was consistent with a bis-phosphorylated Lipid A species possessing two amide-linked 16:0(3-OH) residues, one of which was esterified by a 14:0. The other ion (*m/z* 1138.5) had the same composition and only lacked a phosphate residue. The ion at *m/z* 1444.7 was assigned to a Lipid A species possessing two amide-linked 16:0(3-OH) residues, one of which was esterified by a 14:0, one ester-linked 14:0(3-OH) residue, and two phosphate residues. The ion at *m/z* 1364.7 had the same composition and only lacked a phosphate residue.

Therefore, all the peaks in the spectrum indicated that the secondary fatty acid 14:0 is linked to a *N*-acyl residue.

CONCLUSION

The selectivity of the Lipid A *O*-acyl hydrolysis under mild alkaline conditions suggests the following rate order: primary fatty acid > secondary fatty acid of acyloxyacyl ester > secondary fatty acid of acyloxyacyl amide. This selectivity can be explained considering the substituent effect on the hydrolysis rate of ester groups 1, 2, and 3 (**Scheme 2**). Actually, since the tetrahedral intermediate of alkaline ester hydrolysis is negatively charged, it, and the transition state leading to it, is stabilized by electron-withdrawing substituents (1′, 2′, and 3′). In particular, this property can be expected to decrease for the substituents 1′, 2′, and 3′ of the ether moieties of ester groups 1, 2, and 3, respectively, in the order 1′ > 2′ > 3′, considering the dif-



Scheme 2. Different rate of hydrolysis is explained considering the substituent effect on the hydrolysis rate of ester groups 1, 2, and 3.

ferent inductive effect induced by the electronegative atoms. This effect is higher in 1′ owing to the cooperative effect of the 4-hydroxyl oxygen and 3-amide nitrogen atoms on the sugar ring, than in 2′, due to the ester group, which in turn is higher than that induced by the amide group in 3′ (Scheme 2).

The same electron-withdrawing effects obviously determines that the leaving group ability of the alkoxy moiety of ester 1 is higher than that of ester 2 and this last of ester 3 favors the above ester rate hydrolysis order.

Therefore, the use of mild NH_4OH hydrolysis combined with MALDI-MS analysis of partially degraded Lipid A appears to be a general valuable approach for the determination of complete fatty acid distribution without using other kinds of partial and total degradations of Lipid A. In addition, the work-up of the procedure is simple and easy.

The authors wish to thank O. Holst (Division of Analytical Biochemistry, Research Center Borstel, Center for Medicine and Biosciences) for the gift of lipopolysaccharides from *Klebsiella oxytoca* strain R29 and *Klebsiella pneumoniae* strain R20.

REFERENCES

1. Zähringer, U., B. Lindner, and E. T. Rietschel. 1994. Molecular structure of lipid A, the endotoxic center of bacterial lipopolysaccharides. *Adv. Carbohydr. Chem. Biochem.* **50:** 211–276.
2. Zähringer, U., B. Lindner, and E. T. Rietschel. 1999. Chemical structure of lipid A: recent advances in structural analysis of biologically active molecules. *In* Endotoxin in Health and Disease. D. C. Morrison, H. Brade, S. Opal, and S. Vogel, editors. M. Dekker, Inc., New York. 93–114.
3. Wollenweber, H-W., and E. T. Rietschel. 1990. Analysis of lipopolysaccharide (lipid A) fatty acids. *J. Microbiol. Methods.* **11:** 195–211.
4. Ribeiro, A., Z. Zhou, and C. Raetz. 1999. Multi-dimensional NMR structural analyses of purified Lipid X and Lipid A (endotoxin). *Magn. Reson. Chem.* **37:** 620–630.
5. Zhou, Z., A. Ribeiro, and C. Raetz. 2000. High resolution NMR spectroscopy of lipid A molecules containing 4-amino-4-deoxy-L-arabinose and phosphoethanolamine substituents. *J. Biol. Chem.* **275:** 13542–13551.
6. Que, N., A. Ribeiro, and C. Raetz. 2000. Two dimensional NMR spectroscopy and structure of six lipid A species from *Rhizobium etli* CE3. *J. Biol. Chem.* **275:** 28017–28027.
7. Que, N., S. Lin, R. J. Cotter, and C. Raetz. 2000. Purification and mass spectrometry of six lipid A species from the bacterial endosymbiont *Rhizobium etli*. *J. Biol. Chem.* **275:** 28006–28016.
8. Zhou, Z., S. Lin, R. J. Cotter, and C. Raetz. 1999. Lipid A modifications characteristic of salmonella typhimurium are induced by NH_4VO_3 in *Escherichia coli* K12. Detection of 4-amino-4-deoxy-l-arabinose, phosphoethanolamine and palmitate. *J. Biol. Chem.* **274:** 18503–18514.
9. Therisod, H., M. Monteiro, M. Perry, and M. Caroff. 2001. *Helicobacter mustelae* lipid A structure differs from that of *Helicobacter pylori*. *FEBS Lett.* **499:** 1–5.
10. Kelly, J., H. Masoud, M. Perry, J. Richards, and P. Thibauld. 1996. Separation and characterization of O-deacylated lipooligosaccharides and glycans derived from *Moraxella catarrhalis* using capillary electrophoresis-electrospray mass spectrometry and tandem mass spectrometry. *Anal. Biochem.* **233:** 15–30.
11. Chan, S., and V. Reinhold. 1994. Detailed structural characterization of lipid A: electrospray ionization coupled with tandem mass-spectrometry. *Anal. Biochem.* **218:** 63–73.
12. Johnson, R., G. Her, J. Grabarek, J. Hawiger, and V. Reinhold. 1990. Structural characterization of monophosphoryl lipid A homologs obtained from *Salmonella minnesota* Re595 lipopolysaccharide. *J. Biol. Chem.* **265:** 8108–8116.
13. Qureshi, N., P. Mascagni, E. Ribi, and K. Takayama. 1985. Monophosphoryl lipid A obtained from lipopolysaccharides of *Salmonella minnesota* R595. Purification of the dimethyl derivative by high performance liquid chromatography and complete structural determination. *J. Biol. Chem.* **260:** 5271–5278.
14. Aussel, L., J-R. Brisson, M. B. Perry, and M. Caroff. 2000. Structure of the lipid A of *Bordetella hinzii* ATCC 51730. *Rapid Commun. Mass Spectrom.* **14:** 595–599.
15. Aussel, L., H. Therisod, D. Karibian, M. B. Perry, M. Bruneteau, and M. Caroff. 2000. Novel variation of Lipid A of different *Yersinia* species. *FEBS Lett.* **465:** 87–92.
16. Therisod, H., M. A. Monteiro, M. B. Perry, and M. Caroff. 2001. *Helicobacter mustelae* lipid A structure differs from that *Helicobacter pylori*. *FEBS Lett.* **499:** 1–5.
17. Lindner, B. 2000. Matrix assisted laser desorption/ionisation time-of-flight mass spectrometry of lipopolysaccharides. *In* Bacterial Toxins. O. Holst, editor. Humana Press Inc., Totowa, New Jersey, PA. 311–325.
18. Susskind, M., S. Muller-Loennies, W. Nimmich, H. Brade, and O. Holst. 1995. Structural investigation on the carbohydrate backbone of the lipopolysaccharide from *Klebsiella pneumoniae* rough mutant R20/O1-. *Carbohydr. Res.* **269:** C1–7.
19. Susskind, M., B. Lindner, T. Weimar, H. Brade, and O. Holst. 1998. The structure of the lipopolysaccharide from *Klebsiella oxytoca* rough mutant R29 (O1-/K29-). *Carbohydr. Res.* **312:** 91–95.
20. Silipo, A., R. Lanzetta, D. Garozzo, P. Lo Cantore, N. S. Iacobellis, A. Molinaro, M. Parrilli, and A. Evidente. 2002. Structural determination of the lipid A of the lipopolysaccharide from *Pseudomonas reactans*. A pathogen of the cultivated mushrooms. *Eur. J. Biochem.* **269:** 2498–2505.
21. Adinolfi, M., M. M. Corsaro, C. De Castro, A. Evidente, R. Lanzetta, A. Molinaro, and M. Parrilli. 1996. Analysis of the polysaccharide components of the lipopolysaccharide fraction of *Pseudomonas caryophylli*. *Carbohydr. Res.* **284:** 119–133.
22. Westphal, O., and K. Jann. 1965. Bacterial lipopolysaccharides: Extraction with phenol-water and further applications of the procedure. *Methods Carbohydr. Chem.* **5:** 83–91.