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Chemical Structure of Lipid A from *Helicobacter pylori* Strain 206-1 Lipopolysaccharide

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The chemical structure of a novel lipid A, which was obtained as a major component from lipopolysaccharide of *Helicobacter pylori* strain 206-1, was determined to be a glucosamine β(1-6) disaccharide 1-(2-aminoethyl)phosphate acylated by (*R*)-3-hydroxyoctadecanoic acid and (*R*)-3-(octadecanoyloxy)octadecanoic acid at the 2- and 2′-position, respectively. The absence of a phosphoryl group at the 4′-position and fatty acyl groups at the 3- and 3′-position, and the stoichiometric presence of 2-aminoethyl phosphate at the 1-position are unique features, distinguishing it from the lipid A of enterobacteria.

Key words: *Helicobacter pylori*, lipid A, MS, NMR, structure.

Helicobacter pylori has been frequently isolated from patients with chronic active gastritis, and is associated with duodenal and gastric ulcers in humans (*1-4*). This organism is a Gram-negative, microaerophilic rod and possesses lipopolysaccharide (LPS) located on the outer membrane of the cell surface. Since the bacteria grow in a very low pH environment, the structure of its cell surface may be unique. The chemical and biological properties of *H. pylori* LPS were reported to be distinct from those of the enterobacterial LPS (*5-7*), and in particular, the endotoxic activities were very low (*8, 9*). Recent papers concerning the structure of the O-antigen and the core saccharide region in LPS from *H. pylori* ATCC 43504 (*10*), as well as dyspeptic and asymptomatic strains (*11*) reported that the most striking feature of the O-antigen was the presence of extended chains with fucosylated and non-fucosylated *N*-acetyllactosamine units. With regard to the lipid A moiety in LPS, only a composition analysis was reported (*12*), and its detailed structure has not been determined. Here, we isolated the LPS fraction from *H. pylori* 206-1, which was obtained from a patient, and performed extensive structural analysis of the lipid A molecule.

MATERIALS AND METHODS

Bacteria and Preparation of LPS—*H. pylori* strain 206-1, which was established from a patient at Hyogo College of Medicine, was grown on plates of Brucella agar (Becton Dickinson and Company, Cockeysville, MD, USA) with 7% horse blood and 0.4% Skirrow Supplement (OXOID, Hampshire, UK) and 0.4% Fungizone (Life Technology, New York, NY, USA) in an atmosphere of 5% O_2, 15% CO_2, and 80% N_2 at 37°C for 72 h. The bacterial cells were harvested in PBS and collected by centrifugation at 7,000× *g* at 4°C for 10 min. The collected bacterial cells were then lyophilized.

Chloroform (110 ml) and methanol (220 ml) were added to a suspension of lyophilized *H. pylori* cells (4.4 g) in 110 ml of distilled water. The mixture was stirred at room temperature for 1.5 h, then the solvent was removed by filtration. The filtered cells were washed with methanol and dried *in vacuo* to give delipidated cells (3.7 g) in which free, but not bound, lipids were removed.

The delipidated cells (3.7 g) were suspended in 185 ml of 0.05 M Tris-HCl buffer (pH 7.0). To this suspension, 185 ml of phenol was added and the mixture was stirred at 68°C for 15 min. It was then cooled in an ice bath and centrifuged at 3,000× *g* for 20 min, and the water phase was separated. To the residual mixture, 185 ml of 0.05 M Tris-HCl buffer (pH 7.0) was added, and the mixture was stirred at 68°C for 15 min. After centrifugation under similar conditions to those described above, the water phase was decanted. This process was repeated once more, and the combined water phase was thoroughly dialyzed at 4°C for two days against deionized, microfiltered water prepared with a TORAY PURE LV308 (Toray, Tokyo), then lyophilized to give 0.3 g of crude LPS fraction (8.1 wt% based on the delipidated cells).

Purification of Lipid A—The crude LPS was hydrolyzed with 0.6% acetic acid (0.3 g of LPS in 25 ml of aq. acetic acid) in a sealed tube under nitrogen at 105°C for 2.5 h. The hydrolysate was neutralized with 5% ammonia and made up to 100 ml with distilled water, then 440 ml of mixed solvent composed of $CHCl_3/MeOH/H_2O$/triethylamine (30 : 12 : 2 : 0.01, v/v) was added. After extraction, the

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Abbreviations: CGT, collision activated decomposition gas thickness; CID, collision-induced dissociation; COSY, correlation spectroscopy; ESI-MS, electrospray ionization mass spectrometry; HMBC, heteronuclear multiple-bond correlation spectroscopy; LSIMS, liquid secondary ion mass spectrometry; MS, mass spectrometry; NOESY, nuclear Overhauser enhancement spectroscopy; TLC, thin layer chromatography; TOCSY, total correlation spectroscopy.

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lower organic phase was collected and evaporated to dryness under reduced pressure at 37°C to give a lipid A fraction. The lipid A moiety was purified by silica gel column chromatography (20 g, No. 9385, Merck, Darmstadt, Germany) eluted with $CHCl_3$/MeOH/triethylamine (30 : 5 : 0.1, v/v) and then with $CHCl_3$/MeOH/H_2O/triethylamine (30 : 5 : 0.5 : 0.1, v/v). The eluates were monitored by thin-layer chromatography (Silica gel 60 F_{254}, No. 5715, Merck) developed with $CHCl_3$/MeOH/H_2O/triethylamine (30 : 8 : 0.7 : 0.1) and visualized with anisole/sulfuric acid or ninhydrin reagent. The fractions containing the major component (R_f 0.37) were collected and evaporated to dryness under reduced pressure at 37°C. The residue was suspended in 2 ml of 0.1% aqueous triethylamine at 0°C and lyophilized to give 18 mg of white power (6.0 wt% based on the crude LPS fraction).

Analysis of Component Fatty Acids, Sugars, and Phosphorus—Phosphorus and fatty acids were determined as described in a previous paper (*13*) and references cited therein. No hexose or pentose was detected. Ethanolamine and hexosamine were analyzed by an amino acid analyzer (655A, Hitachi, Tokyo) (*14*). The configuration of the 3-hydroxyoctadecanoic acid was determined by TLC according to the method developed in our laboratory after condensation with N^δ-benzyloxycarbonyl-(*S*)-ornithine benzyl ester. In brief, the purified lipid A (2.6 mg) was hydrolyzed overnight with 6 M hydrochloric acid at 105°C. Liberated fatty acids were extracted with hexane. After removal of the solvent by passing a stream of nitrogen, 462 μl of a chloroform solution containing N^δ-benzyloxycarbonyl-(*S*)-ornithine benzyl ester *p*-toluensulfonate (3.0 mg, 8.4 μmol), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (1.6 mg, 8.4 μmol), and triethylamine (2.3 μl, 17 μmol) was added. The mixture was stirred overnight at room temperature and directly analyzed by TLC. The plate (Silica gel 60 F_{254}, No. 5715, Merck) was developed with toluene/ethyl acetate (1 : 1, v/v) and visualized with HBr/ninhydrin. The lipid fraction obtained by 6 M HCl hydrolysis of *Escherichia coli* Re LPS, racemic 3-hydroxyoctadecanoic acid, and (*S*)- and (*R*)-3-hydroxytetradecanoic acid were condensed similarly with N^δ-benzyloxycarbonyl-(*S*)-ornithine benzyl ester and the products were used as references.

Spectrometric Methods—NMR spectroscopy: ^{1}H and ^{31}P NMR spectra were measured on a Varian Unity 600 spectrometer at 600 and 242 MHz at 25°C, respectively. The chemical shifts of ^{1}H and ^{31}P were referenced to an internal standard of tetramethylsilane at 0 ppm and to an external standard of triphenyl phosphate at −18 ppm, respectively. The sample was dissolved in $CDCl_3$/MeOH-d_4/pyridine-d_5 (1 : 1 : 0.01, v/v) or in $CDCl_3$/MeOH-d_4 (1 : 1, v/v) at the concentration of 3 mg/ml. For the measurement of ^{1}H-^{1}H TOCSY, the WETTOCSY pulse sequence was used. The COSY, NOESY, TOCSY, and HMBC spectra were typically recorded with 6,000 Hz spectral width in both dimensions and 512 data points in f2 and 2,048 data points in f1 with 16 (COSY, NOESY, and TOCSY) or 128 (HMBC) scans at each increment. The mixing times were 1,300 ms for NOESY and 80 ms for TOCSY. All data processing was done with FELIX v. 95 (MSI, San Diego, CA, USA). The time domain data were multiplied with a squared sine-bell function for TOCSY.

Mass spectrometry: ESI-MS and ESI-MS/MS were obtained with an API III plus mass spectrometer (PE SCIEX, Thornhill, Ontario, Canada) equipped with an ion-spray source (ion-spray voltage: −5 kV; nebulizer gas: nitrogen; drying gas: nitrogen). Samples were typically dissolved in chloroform/MeOH (1 : 1, v/v) at a concentration of 10 μg/ml and introduced at a flow rate of 5 μl/min. The MS/MS analysis was performed using argon (CGT: 3×10^{15} molecule/cm^2) as the collision gas at a 70 eV collision energy.

LSIMS was carried out on a CONCEPT 1H (Shimadzu/Kratos, Kyoto) mass spectrometer in the negative mode (acceleration voltage: 8 kV; ion beam: cesium at 15 keV). *m*-Nitrobenzyl alcohol was used as the matrix. Linked scan (LSIMS/MS) spectra were obtained after CID with air introduced into the collision cell.

RESULTS AND DISCUSSION

According to the procedure described in "MATERIALS AND METHODS," LPS of *H. pylori* strain 206-1 was extracted, and its major lipid A component (R_f=0.37) was purified after acetic acid hydrolysis of the LPS. From analysis of the chemical composition, the purified lipid A was found to contain glucosamine (1.6 μmol/mg), octadecanoic acid (0.77 μmol/mg), 3-hydroxyoctadecanoic acid (1.5 μmol/mg), and phosphorus (0.71 μmol/mg) in a molar ratio of approximately 2 : 1 : 2 : 1. The configuration of the 3-hydroxy acid was deduced by TLC analysis of the coupling product with the (*S*)-ornithine derivative. The coupling compound derived from the lipid A gave a spot which was identical to the slower-moving product from racemic 3-hydroxyoctadecanoic acid. The present 3-hydroxyoctadecanoic acid was determined to have (*R*)-configuration, since authentic (*R*)-3-hydroxy acids always move more slowly than the (*S*)-enantiomers. The purified lipid A was positive to ninhydrin reagent on TLC, indicating the existence of amino group(s). In accordance with this observation, ethanolamine was detected in the amino acid analysis. In the ^{1}H-^{31}P HMBC spectrum of the purified lipid A measured in $CDCl_3$/MeOH-d_4/pyridine-d_5 (1 : 1 : 0.01, v/v), cross-peaks were observed between the two ^{1}H-signals at 4.23 and 5.53 ppm and the ^{31}P-signal at −1.7 ppm. From cross-peaks observed in the ^{1}H-^{1}H COSY spectrum measured in the same solvent system (Fig. 1a), the ^{1}H-signal at 4.23 ppm was assigned to a methylene proton coupled with the other proton of the same methylene group at 4.11 ppm, both of the protons coupling with other methylene protons at 3.20 and 3.25 ppm. These facts indicate the presence of an aminoethyl phosphate structure.

The ^{1}H-signal at 5.53 ppm, which couples with ^{31}P as described above, was assigned to the proton at the glycosidic position carrying the aminoethyl phosphate. The signal was observed as a broad peak in the spectra measured in $CDCl_3$/MeOH-d_4/pyridine-d_5 (1 : 1 : 0.01, v/v). In the spectrum measured in $CDCl_3$/MeOH-d_4 (1 : 1, v/v), however, the corresponding proton appeared as a typical double doublet signal at 5.48 ppm ($^3J_{H1,H2}$=3.4 Hz, $^3J_{H1,P}$=8.6 Hz). The coupling constant value of $^3J_{H1,H2}$ proved the α-anomeric configuration. From the anomeric proton at 5.53 ppm, the signals of all protons of the reducing glucosamine residue were assigned by using ^{1}H-^{1}H COSY and ^{1}H-^{1}H TOCSY spectra (Fig. 1) measured in $CDCl_3$/MeOH-

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d_4/pyridine-d_5 (1 : 1 : 0.01, v/v). The 1D ¹H NMR spectrum measured in the same solvent system showed a doublet peak at 4.40 ppm ($^3J_{H1',H2'}$=8.2 Hz), which was assigned to the other anomeric proton at H1′ of the β-linked nonreducing glucosamine. From this doublet peak, all protons of the nonreducing glucosamine were assigned by means of ¹H-¹H COSY and ¹H-¹H TOCSY spectra (Fig. 1). In the ¹H-¹H NOESY spectrum measured in $CDCl_3$/MeOH-d_4 (1 : 1, v/v), three cross-peaks were observed with the anomeric proton at H1′ (4.37 ppm): two were with the methylene protons at H6 (3.82 and 4.03 ppm), and the other with the methine proton at H5 (4.21 ppm). No other inter-residual cross-peak with H1′ was detected. These NOE data clearly demonstrated the 1-6 linkage. The peaks of H2 and H2′ appeared at 3.89 and 3.54 ppm (Fig. 1), respectively, proving *N*-acyl substitutions. The methine proton signals of H3, H4, H3′, and H4′ were found between 3 and 4 ppm (Fig. 1), suggesting no acyl or phosphoryl substitution at the hydroxy groups of these positions.

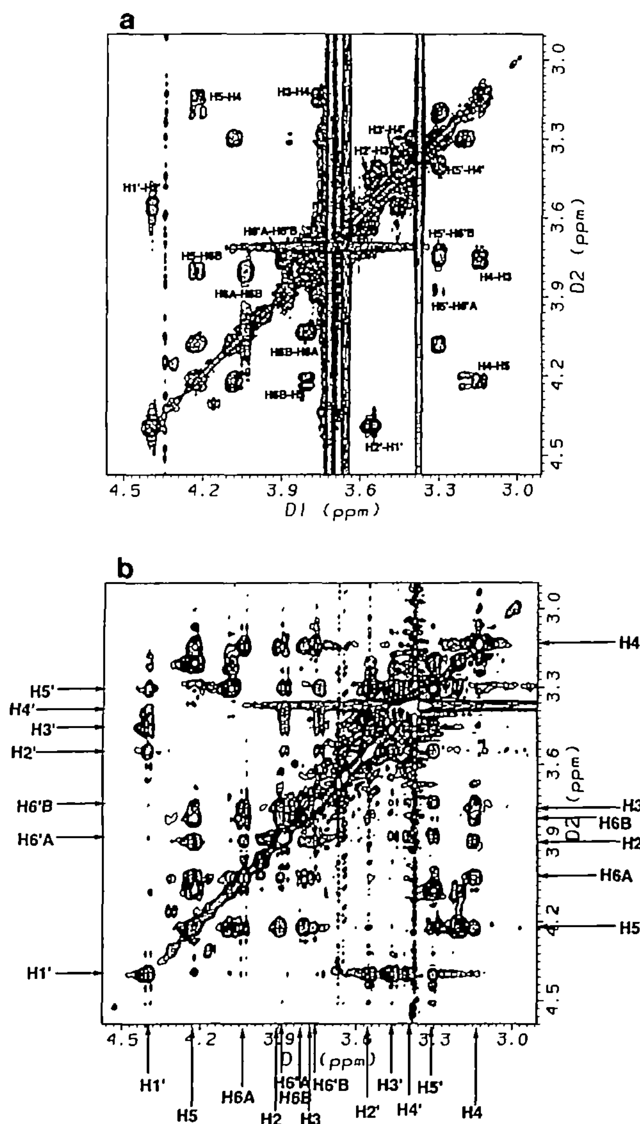

Fig. 1. **¹H-¹H COSY (a) and ¹H-¹H TOCSY (b) spectra of the purified lipid A from *H. pylori* strain 206-1.** Solvent: $CDCl_3$/MeOH-d_4/pyridine-d_5 (1 : 1 : 0.01, v/v).

Similarly, the chemical shift values of methylene protons at H6′ (3.88 and 3.74 ppm) indicated that the 6′-hydroxy group was neither acylated nor phosphorylated. Thus, the structure of the hydrophilic backbone of the lipid A was elucidated to be a β(1-6) disaccharide of glucosamine with an aminoethyl phosphoryl group linked to the glycosidic position.

In the ¹H-¹H COSY NMR spectrum measured in $CDCl_3$/MeOH-d_4/pyridine-d_5 (1 : 1 : 0.01, v/v), two β-methine proton signals of 3-oxyoctadecanoic (β-oxyoctadecanoic) acids were observed at 3.97 and 5.24 ppm, respectively. The former coupled with α- and γ-methylene protons at 2.31 and 1.49 ppm, and the latter with those at 2.50 and 1.60 ppm. The former methine signal was attributed to a 3-hydroxyoctadecanoyl group and the latter to a 3-(octadecanoyl)oxydecanoyl group. The locations of these acyl groups and the overall structure of lipid A were determined mass-spectrometrically as follows.

In the negative mode ESI-MS and LSIMS, the pseudo molecular ion peak $(M-H)^-$ was observed at *m/z* 1,293. The negative mode tandem ESI-MS/MS or LSIMS/MS analysis of this ion peak gave a fragment ion at *m/z* 583.5 or 584 (Fig. 2). This indicates that the (*R*)-3-hydroxyoctadecanoyl group is attached to the 2-amino group of the reducing glucosamine (Fig. 3). Fragment ions at *m/z* 612, 566, and 463 found in LSIMS/MS are additional evidence for the position of the (*R*)-3-hydroxyoctadecanoyl group (*15*). Consequently, the (*R*)-3-(octadecanoyloxy)octadecanoyl group was deduced to be at the 2′-amino group.

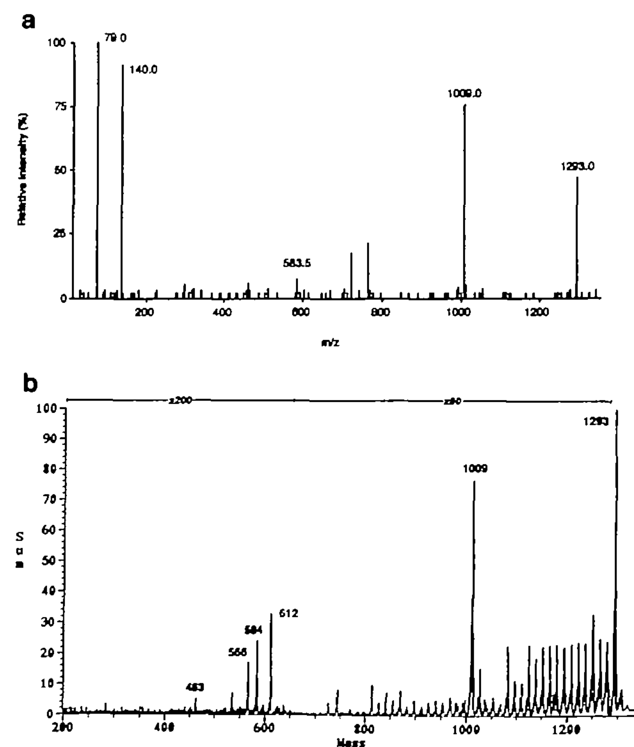

Fig. 2. **ESI-MS/MS (a) and LSIMS/MS (b) spectra of the purified lipid A from *H. pylori* strain 206-1.** Matrix of LSIMS/MS: *m*-nitrobenzyl alcohol. The conditions of tandem MS/MS analyses were described in "MATERIALS AND METHODS." The precursor ion at *m/z* 1,293 [$(M-H)^-$] was employed in both MS/MS analyses.

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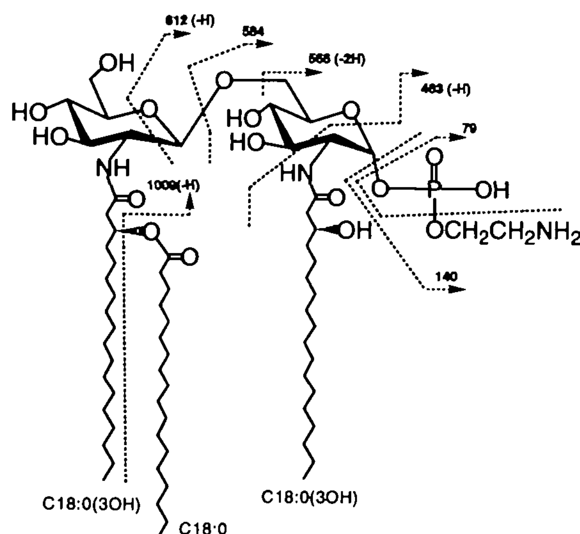

Fig. 3. **Proposed structure of lipid A from *H. pylori* strain 206-1.** Arrows and numbers represent the fragmentation and the corresponding m/z observed in ESI-MS/MS and/or LSIMS/MS.

Therefore, the structure of the lipid A from *H. pylori* strain 206-1 was proposed to be as shown in Fig. 3. The negative-mode, high-resolution LSIMS gave a pseudo-molecular ion $(M-H)^-$ at m/z 1,292.91953, which was in a good agreement (Δ 1.99 mamu) with this structure. The fragment ions at m/z 149 and 79 detected in the ESI-MS/MS corresponded to $P(O)OH(OCH_2CH_2NH_2)O$- and $P(O)OHO$-, respectively, confirming the presence of an aminoethyl phosphoryl group. The fragment ion at m/z 1,009 found in both tandem MS/MS analyses indicates β-elimination of octadecanoic acid, supporting the presence of a β-acyloxyacyl moiety in the lipid A (*16*).

The stoichiometric presence of an aminoethyl group may contribute to the intramolecular neutralization of the negative charge of the phosphate group in this lipid A. A preliminary *in vacuo* MOPAC calculation gave a seven-membered ring *via* hydrogen bonding between the amino and phosphate groups (data not shown). Since LPS is a major component of the outer membrane of Gram-negative bacteria, the unique structure of lipid A of *H. pylori* described in this report might directly reflect the ability of this bacteria to grow in the very low pH condition in the stomach.

We have proposed here a novel structure of the major lipid A from *H. pylori* strain 206-1. During the purification, we also obtained another minor component (R_f=0.42). The ESI-MS analysis of this minor component suggested the presence of an additional 3-hydroxyhexadecanoic acid. Interestingly, the ratio of the two lipid A components varied between culture batches. This may be due to the different ratio of rough- and smooth-form LPS in individual cultures, because the SDS-PAGE profiles of LPS of *H. pylori* strain 206-1 were not always the same. The major lipid A isolated in the present work might, therefore, correspond to the lipid A moiety of either rough or smooth form LPS of this bacteria. The structural variety of the lipid A moiety of *H. pylori* was also mentioned by Moran and Aspinall (*17*). According to recent reports by them and by Amano (*18*), *H. pylori* may have a variety of structures of both O-antigen polysaccharide and lipid A moiety in LPS, depending on the strain and the growth conditions. Further extensive study is needed to understand the general structural features of lipid A and LPS of this bacteria.

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REFERENCES

1. Warren, J.R. and Marshall, B. (1983) Unidentified curved bacilli on gastric epithelium in active chronic gastritis. *Lancet* **I**, 1273-1275
2. Buck, G.E., Gourley, W.K., Lee, W.K., Subramanyan, K., Latimer, J.M., and DiNuzzo, A.R. (1986) Relation of *Campylobacter pyloridis* to gastritis and peptic ulcer. *J. Infect. Dis.* **153**, 664-669
3. O'Connor, H.J. (1994) The role of *Helicobacter pylori* in peptic ulcer disease. *Scand. J. Gastroenterol.* **201**, 11-15
4. Goodwin, C.S. (1995) The impact of microbiological discoveries such as *Helicobacter pylori* on gastroenterology, and the implications for microbiologists. *Med. Microbiol. Lett.* **4**, 155-164
5. Conrad, R.C., Kist, M., Schlecht, S., and Galanos, C. (1992) Extraction and biochemical analyses of *Helicobacter pylori* lipopolysaccharide. *Curr. Microbiol.* **24**, 165-169
6. Geis, G., Leying, H., Suerbaum, S., and Opferkuch, W. (1990) Unusual fatty acid substitution in lipids and lipopolysaccharides of *Helicobacter pylori*. *J. Clin. Microbiol.* **28**, 930-932
7. Cunningham, M.D., Seachord, C., Ratcliffe, K., Bainbridge, B., Aruffo, A., and Darveau, R.P. (1996) *Helicobacter pylori* and *Porphyromonas gingivalis* lipopolysaccharide are poorly transferred to recombinant soluble CD14. *Infect. Immun.* **64**, 3601-3608
8. Muotiala, A., Helander, I.M., Pyhälä, L., Kosunen, T.U., and Moran, A.P. (1992) Low biological activity of *Helicobacter pylori* lipopolysaccharide. *Infect. Immun.* **60**, 1714-1716
9. Birkholz, S., Knipp, U., Nietzki, C., Adamek, R.J., and Opferkuch, W. (1993) Immunological activity of lipopolysaccharide of *Helicobacter pylori* on human peripheral mononuclear blood cells in comparison to lipopolysaccharides of other intestinal bacteria. *FEMS Immunol. Med. Microbiol.* **6**, 317-324
10. Aspinall, G.O., Monteiro, M.A., Pang, H., Walsh, E.J., and Moran, A.P. (1996) Lipopolysaccharide of the *Helicobacter pylori* type strain NCTC 11637 (ATCC 43504): Structure of the O antigen and core oligosaccharide regions. *Biochemistry* **35**, 2489-2497
11. Aspinall, G.O. and Monteiro, M.A. (1996) Lipopolysaccharides of *Helicobacter pylori* strains P466 and MO19: Structures of the O antigen and core oligosaccharide regions. *Biochemistry* **35**, 2498-2504
12. Mattsby-Baltzer, I., Mielniczuk, Z., Larsson, L., Lindgren, K., and Goodwin, S. (1992) Lipid A in *Helicobacter pylori*. *Infect. Immun.* **60**, 4383-4387
13. Suda, Y., Tochio, H., Kawano, K., Takada, H., Yoshida, T., Kotani, S., and Kusumoto, S. (1995) Cytokine-inducing glycolipids in the lipoteichoic acid fraction from *Enterococcus hirae* ATCC 9790. *FEMS Immunol. Med. Microbiol.* **12**, 97-112
14. Fukase, K., Kitazawa, M., Sano, A., Shinbo, K., Horimoto, S., Fujita, H., Kubo, A., Wakamiya, T., and Shiba, T. (1992) Synthetic study of peptide antibiotic Nisin. V. Total synthesis of Nisin. *Bull. Chem. Soc. Jpn.* **65**, 2227-2240
15. Ogawa, T. (1993) Chemical structure of lipid A from *Porphyromonas* (*Bacteroides*) *gingivalis* lipopolysaccharide. *FEBS Lett.* **332**, 197-201
16. Chan, S. and Reinhold, V.N. (1994) Detailed structural characterization of lipid A: Electrospray ionization coupled with tandem mass spectrometry. *Anal. Biochem.* **218**, 63-73

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17. Moran, A.P. and Aspinall, G.O. (1996) Unique structural and biological features of *Helicobacter pylori* LPS. *J. Endotoxin Res.* (*Abstract of 4th Conference of the International Endotoxin Society, Nagoya, Japan*) **3**, S-4

18. Amano, K. (1996) Reactivity of *H. pylori* LPS with monoclonal antibodies for Lewis antigens. Evening Seminar for "*Helicobacter pylori* and LPS" at the 4th Conference of the International Endotoxin Society, Nagoya, Japan

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