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Structural characterization of lipid A obtained from *Pantoea agglomerans* lipopolysaccharide

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Abstract

Lipopolysaccharide isolated from *Pantoea agglomerans* showed higher priming and triggering activities for macrophages in terms of tumor necrosis factor production than other lipopolysaccharides. To identify the difference in biological activities of lipopolysaccharide of *Pantoea agglomerans* from other lipopolysaccharides on the basis of structure, we determined the structure of the lipid A part, which is the biological center of lipopolysaccharide, by quantitative analysis, nuclear magnetic resonance spectroscopy and mass spectrometry. Lipopolysaccharide of *Pantoea agglomerans* is constructed with at least two kinds of lipid A of different levels of acylation. One is of the same type as that of *Escherichia coli* with hexa-acyl lipid A and the other is the *Salmonella minnesota* type with hepta-acyl lipid A.

Keywords: *Pantoea agglomerans*; Lipopolysaccharide; Lipid A structure

1. Introduction

Bacterial lipopolysaccharide (LPS) shows numerous biological activities through macrophage activation [1]. These activities depend primarily on the chemical structure of lipid A, which is the active center of LPS [1–3]. Lipid A from various Gram-negative bacterial origins usually consists of an amino sugar backbone carrying fatty acids and phosphates. Full biological activity requires a molecule containing two hexosamine residues, two phosphoryl groups, and six fatty acids in a defined location, as is present in *Escherichia coli* lipid A. Of the disaccharide compounds, those with only one phosphoryl group were significantly less active (up to 100-fold) than bisphosphorylated compounds. Fatty acids were found to be of great importance in the expression of lipid A bioactivities and their number and location, and most likely their chain length and stereochemistry, have been confirmed to play significant roles [1–3].

We found that low molecular mass LPS from the Gram-negative bacterium *Pantoea agglomerans*

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(LPSp) showed higher macrophage-activating activity in terms of production of tumor necrosis factor (TNF) than did *E. coli* LPS. LPSp showed preventive and curative effects for various intractable diseases (ulcers, diabetes, hyperlipidemia, and others) [4–8], and actually showed antitumor effects without severe side effects, when administered intradermally in a clinical study in human patients [9].

From these observations, we hypothesized that the strong therapeutic effects of LPSp could be attributed to the structure of its lipid A. Thus, to identify the difference in biological activity of LPSp from other LPSs on the basis of structure, we analyzed the structure of lipid A derived from *P. agglomerans* LPS in this work. The structure of the lipid A moiety from *P. agglomerans* was determined by component analysis and by NMR spectroscopy and mass spectrometry [10] of dimethyl derivatives of lipid A.

2. Materials and methods

2.1. Bacterium and bacterial LPS

Details of the isolation of *P. agglomerans*, culture conditions and purification method of LPS from this organism were described in a preceding paper [4]. Ten liter scale bacterial culture was done using a 10 liter fermenter (Model MDL 1000 Desk-top Fermenter, B.E. Marubishi Co., Ltd).

2.2. Purification of lipid A from P. agglomerans

Preparation of lipid A was done by mild acid hydrolysis (0.1 N HCl, at 100°C for 90 min) of crude LPSp. From the initial material of 500 g of wet bacterial mass, 1.4 g of crude lipid A preparation was obtained. Further purification of lipid A from the crude preparation was done by silica gel column chromatography. Samples were applied to the silica gel column (silica gel 130 g; Fuji gel 2061) and eluted with solvents ($CHCl_3$-CH_3OH-H_2O-$(CH_3$-$CH_2)_3$-N (87:12:1:0.2, 85:14:1:0.2, 83:16:1:0.2, v/v). Hepta-acyl lipid A fraction (fraction C) and hexa-acyl lipid A fraction (fraction D) were obtained and used for chemical component analyses. Hepta-acyl lipid A fraction was also methylated using diazomethane and purified chromatographically to obtain a pure compound **1** (30 mg) as an amorphous powder for spectral analysis.

2.3. Analytical methods

Hexosamine was measured by the Elson-Morgan method using *N*-acetyl-glucosamine as a standard [4]. Phosphate was measured by the Chen-Toribara method [4]. Fatty acids were analyzed by high performance liquid chromatography (HPLC). Briefly, total fatty acids were released from lipid A by acidic hydrolysis (6 M HCl, 4 h, 100°C). Free fatty acids were extracted with $CHCl_3$. Pentadecanoic acid was

Table 1
Chemical composition of purified lipid A samples from *Pantoea agglomerans*

Sample	Constituents[a]								
	Glucosamine		Phosphoric acid		Fatty acids[c]				
	Molar ratio	wt%	Molar ratio	wt%	Molar ratio				wt%
					3-OH-14:0	12:0	14:0	16:0	
Lipid A fraction C	1.9	15.3	1.0	4.5	4.0	1.1	1.0	1.1	68.0
Lipid A fraction D	1.7	16.0	1.0	5.4	4.0	0.2	1.0	1.0	59.0
Synthetic lipid A[b]	1.7	15.3	2.1	10.3	4.0	0.0	1.2	1.1	67.0

[a]wt%: Expressed in % weight per lipid A molecule ratio. The chemical compositions of the three lipid As are expressed on the basis of molar ratios calculated from the molecular weight of each lipid A triethylamine salt (lipid A fraction C: 2200 Da, lipid A fraction D: 1900 Da, synthetic lipid A: 2000 Da). The fatty acid compositions of the three lipid As are expressed on the basis of the molar ratios calculated from 3-OH-14:0 as 4 mol per lipid A molecule.

[b]Synthetic lipid A (LA-15-PP(506)); Diphosphoryl hexa-acyl lipid A.

[c]Fatty acids were analyzed by HPLC and were identified and quantified by comparing retention times and peak areas with those of fatty acid standards. 3-Hydroxytetradecanoic acid was identified and quantified by comparison of the retention time with that of components separated from synthetic lipid A (LA-15-PP(506)) used as the standard lipid A preparation.

added as an internal standard and the mixture was dried. Dried samples were treated with 0.01 M KOH/CH_3OH at 60°C, then 2 mM 18-crown 6-ether together with 20 mM *p*-bromophenacyl bromide acetonitrile solution was added and reacted at 80°C for 30 min. *p*-Bromophenacyl ester derivatives thus prepared were analyzed with a reverse phase HPLC system.

2.4. Nuclear magnetic resonance spectroscopy

Proton nuclear magnetic resonance (^{1}H-NMR) spectra of compound **1** (30 mg ml^{-1}) were recorded with a GSX-500 (JEOL, Ltd., Tokyo, Japan) in $CDCl_3$ at 35°C as parts per million downfield from tetramethylsilane (TMS). Carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectra of compound **1** (30 mg ml^{-1}) were recorded with a JEOL GSX-500 spectrometer in $CDCl_3$ at 35°C (internal TMS). Abbreviations used are s = singlet, d = doublet, t = triplet, br = broad, m = multiplet, dd = doublet of doublets, q = quartet.

3. Results and discussion

3.1. Chemical component analyses of lipid A

The results of component analyses are shown in Table 1. Purified lipid A preparations of fractions C and D showed the existence of about 1 mol phosphate in a lipid A molecule. Hexosamine was detected at about 2 mol per molecule. Fraction C was composed of dodecanoic acid (12:0), tetradecanoic acid (14:0), hexadecanoic acid (16:0) and 3-hydroxytetradecanoic acid [14:0(3-OH)] in a ratio of approximately 1:1:1:4, and fraction D showed 12:0, 14:0, and 14:0(3-OH) in a ratio of approximately 1:1:4.

Analysis of amide-linked fatty acids [10] showed that compound **1** had hexadecanoyloxytetradecanoyl (3-*O*-(16:0)-14:0) and dodecanoyloxytetradecanoyl (3-*O*-(12:0)-14:0) residues (Fig. 1a). Fraction D had only one (3-*O*-(12:0)-14:0) residue (Fig. 1b).

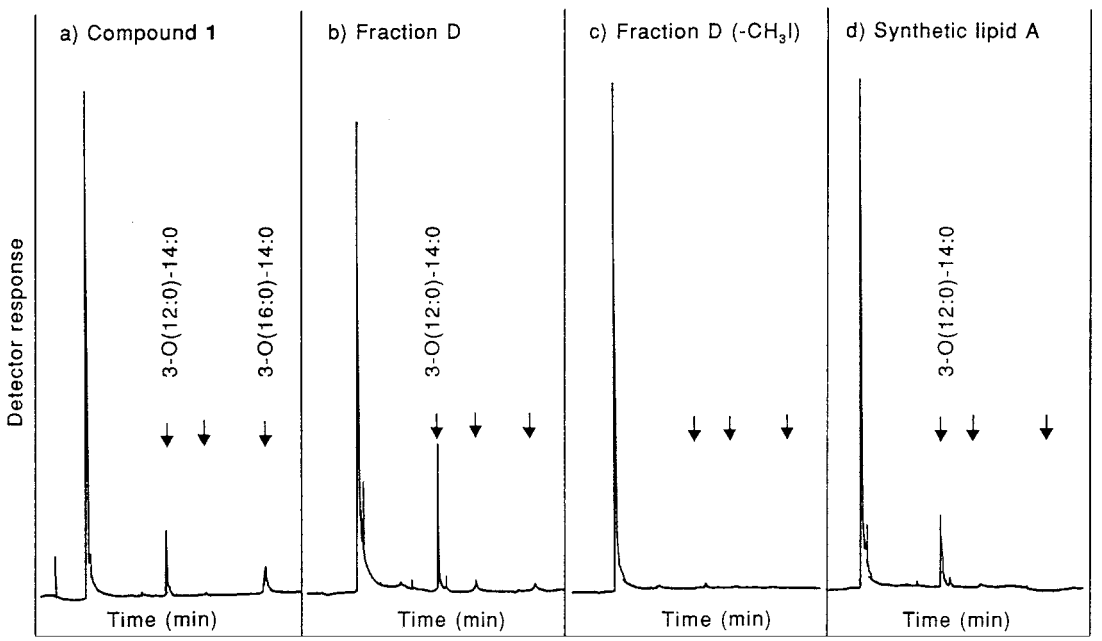

Fig. 1. Gas chromatograms of acyl residues liberated from *P. agglomerans* lipid A samples by amide-linkage specific hydrolysis with CH_3I/Ag^+ and mild acid [11]. A gas chromatograph (Hitachi G-3000) was used for separations on a capillary column (Supelco, SP-2380, 0.25 mm × 30 m) using nitrogen as carrier gas (12.6 ml/min) at 230°C. a: Compound 1; b: fraction D; c: fraction D (-CH_3I) treated with silver salts in the absence of CH_3I; d: synthetic lipid A (*E. coli* type hexa-acyl lipid A). Arrows indicate the retention time (Rt) of each reference methyl ester, 3-*O*-(12:0)-14:0 (Rt: 7.50 min), 3-*O*-(14:0)-14:0 (Rt: 9.89 min), 3-*O*-(16:0)-14:0 (Rt: 13.40 min). Acyloxyacyl methylester standards were prepared in our laboratory.

Table 2
NMR spectral data of the sugar moieties of compound **1**

Reducing glucosamine moiety			Non-reducing glucosamine moiety	
No.	Proton[a]	Carbon[b]	Proton[a]	Carbon[b]
1	5.15 (d, $J=3$ Hz)	91.5	5.10 (d, $J=8$ Hz)	100.2
2	4.17 (dt, $J=3$, 9.5 Hz)	51.8	3.61 (br q, $J=9$ Hz)	55.3
3	5.15 (t, $J=9.5$ Hz)	74.6	5.38 (t, $J=9.5$ Hz)	72.2
4	3.50 (t, $J=9.5$ Hz)	69.1	4.38 (q, $J=9.5$ Hz)	73.3
5	4.03 (dd, $J=9.5$, 6.5 Hz)	71.2	3.51 (br d, $J=9$ Hz)	74.6
6	3.80 (dd, $J=12$, 6.5 Hz)	68.4	3.87 (2H, m)	60.6
	4.08 (br d, $J=9$ Hz)			
NH	6.17 (d, $J=9$ Hz)		6.48 (br d, $J=8$ Hz)	
OME			3.75 (6H, d, $J=11$ Hz)	54.8 (d):55.0 (d)

[a]Chemical shift value (δ) with the coupling constants of protons shown in parentheses.
[b]Chemical shift value (δ) of carbon atoms.

3.2. Spectrometric analysis

The structure of the lipid A backbone, the distribution pattern of acyl residues, the positions of free hydroxyl groups and the attachment sites of phosphate were analyzed using ^{1}H-, ^{13}C-NMR spectroscopy and fast atom bombardment (FAB) mass spectrometry [9]. Chemical shift values of the sugar portion of compound **1** determined by NMR analysis are shown in Table 2. The ^{1}H-NMR spectrum of this compound shows the presence of two phosphomethoxyl groups [δ 3.75 (d, $J=11$ Hz)], two amide protons [δ 6.17 (d, $J=9$ Hz); 6.48 (br d, $J=8$ Hz)], two anomeric protons [δ 5.10 (d, $J=8$ Hz); 5.15 (d, $J=3$ Hz)] and long chain alkyl groups [δ 0.88 (t, $J=7$ Hz, CH_3); 1.27 (br s, CH_2)]. In the ^{13}C-NMR spectrum, seven carbon signals due to ester carbonyl or amide carbonyl carbon were observed at δ 170.0; 170.2; 171.0; 173.2; 173.3; 173.4; 174.3, suggesting that compound **1** has seven acyl residues. Assignment of the proton signals of the glucosamine moieties was made starting from each anomeric proton signal in the 'proton and proton correlated spectroscopy' (^{1}H-^{1}H COSY) spectrum. The proton signals of H-2 to H_2-6 can be assigned as shown in Table 3. From these chemical shifts, C-1 and C-4 of the reducing glucosamine moiety and C-6 of the non-reducing glucosamine moieties are not acylated and C-4 of the non-reducing glucosamine moiety was observed as a quartet ($J=9.5$ Hz), suggesting that C-4 is phospho-esterified.

All carbon signals of the sugar moieties were assigned as shown in Table 2 from the proton and carbon-13 correlated spectroscopy (^{1}H-^{13}C COSY) spectrum, position C-6 (δ 68.4) of the reducing glucosamine moiety is shown as not being acylated. H-4 of the non-reducing glucosamine moiety, which was found to be shifted downfield by ca. 0.88 ppm from that of reducing glucosamine, is shown as phospho-esterified.

The anomeric protons of the disaccharide were determined by the coupling constant. The H-1 of the reducing glucosamine moiety showed a signal at δ 5.15 with coupling constant $J=3$ Hz; the anomeric configuration was therefore determined to be α. The anomeric proton signal of the non-reducing glucosamine moiety (δ 5.10) displayed a larger coupling constant, $J=8$ Hz, so that the anomeric configuration was assigned as β.

The positive FAB-MS spectrum of compound **1**

Table 3
Summary of mass peaks observed in the positive first atom bombardment mass spectroscopy of diazomethane treated fraction C

Parent ion (*m/z*)	Fragment ion (*m/z*)	Cleaved residue[a]	Form
2008	1115	893	reducing GlcN
	915	G and 200	C12:0
	789	G and 200 and 126	C12:0 and $(MeO)_2PO_2H$

[a]The symbols G and C12:0 indicate reducing glucosamine unit (893) and dodecanoic acid, respectively.

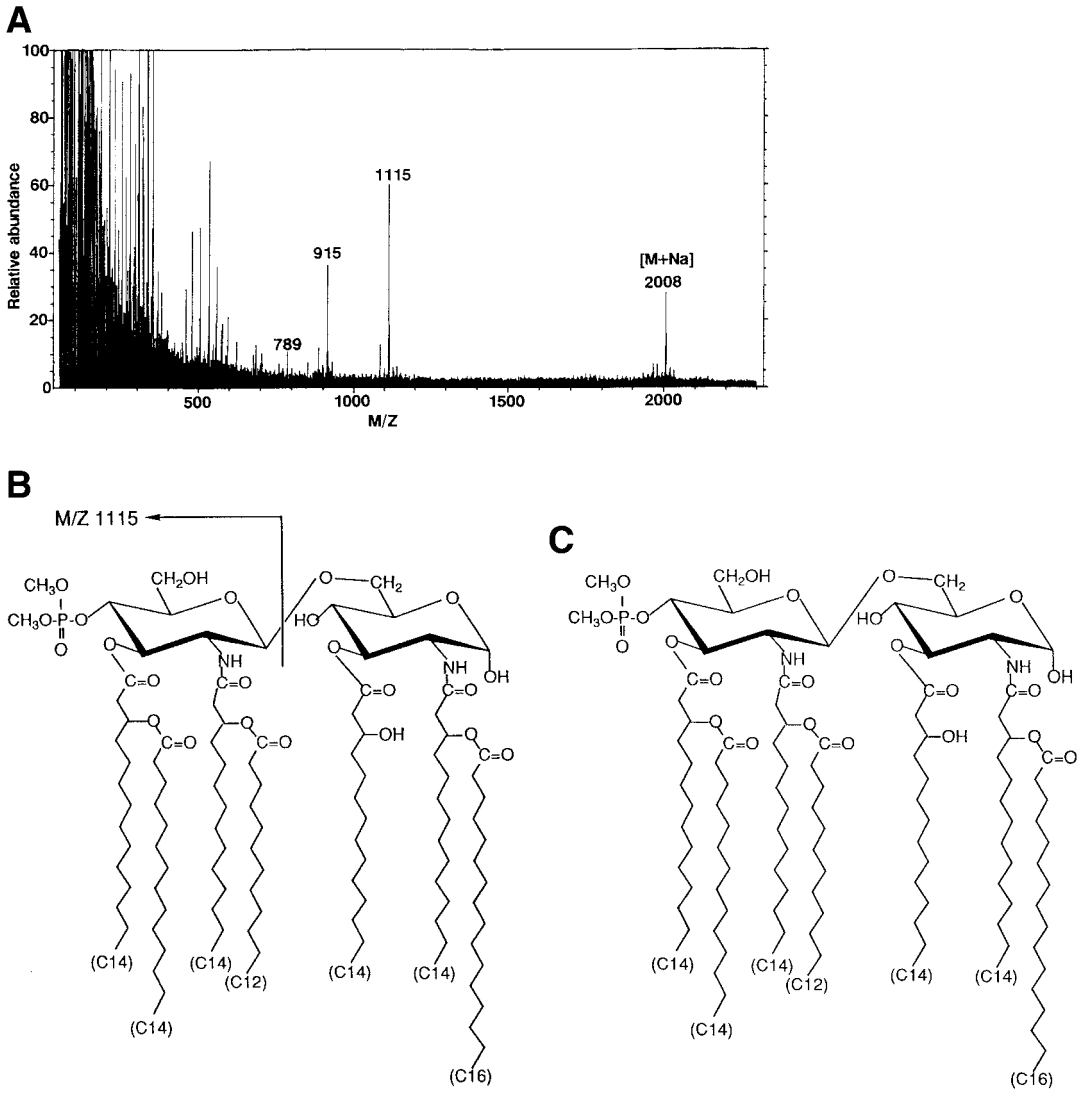

Fig. 2. A: Positive FAB mass spectrum of purified dimethyl lipid A from *P. agglomerans*. B: Proposed structure and degradation pattern. C: Proposed structure of dimethyl hepta-acyl lipid A from *P. agglomerans*. Positive FAB-MS spectrometry was performed with a JMS-SX102 (JEOL, Ltd., Tokyo, Japan) mass spectrometer. *m*-Nitrobenzyl alcohol was used as the matrix.

shows the pseudo molecular ion peaks at *m/z* 2008 being compatible with the molecular formula $C_{112}H_{211}O_{23}N_2P$+Na (Fig. 2A). The fragment ion peak at *m/z* 1115 which is derived from the cleavage of the glycosidic linkage revealed the presence of a 3-*O*-(12:0)-14:0 and a 3-*O*-(14:0)-14:0 residue in the non-reducing glucosamine moiety (Fig. 2B). The fragment ion peak at *m/z* 915 is believed to be derived from the cleavage of the glycosidic linkage and the simultaneous loss of 200 Da corresponding to cleavage of a 12:0 residue accompanied by H-transfer to 12:0 (Table 3). Therefore, the 14:0 (3-OH) residue is thought to be attached to C-3 of the reducing glucosamine moiety. Based on the above results together with the analyses of chemical components, the structure of purified dimethyl-monophosphoryl hepta-acyl lipid A from *P. agglomerans* has been proposed as the structure illustrated in Fig. 2C.

This structure has now been confirmed to be the

same as that of *Salmonella* type 4′-monophosphoryl hepta-acyl lipid A [2,3]. Another type, hexa-acyl lipid A, has been shown to have the same structure as *E. coli* type 4′-monophosphoryl hexa-acyl lipid A [2].

Comparison with the structure-function relationship of lipid A, which has the typical structure of *E. coli* type lipid A backbone, demonstrated that activities differed depending on: (1) the number of phosphoryl and acyl residues, (2) the substituted site of phosphoryl and acyl residues and (3) the chain length of acyl residues [11]. The *E. coli* type hexa-acyl lipid A from *P. agglomerans* showed the highest activity so far known, including endogenous TNF induction [2], whereas the *Salmonella* type hepta-acyl lipid A activity was slightly lower [2]. Thus, the beneficial therapeutic effects of LPSp, which were not observed with other LPS, are not explainable by the structure of its lipid A. We have shown that LPSp is composed mainly of 5 kDa low molecular mass LPS and, to a lower extent, of 30–60 kDa high molecular mass LPS, whereas the *E. coli* LPS tested was composed largely of high molecular mass LPS. The results of several LPS structure-function studies have shown that polysaccharide chain length and composition of LPS can significantly influence both its physicochemical properties and its biological activity [1].

Thus, there is a possibility that composition and polysaccharide chain length of LPSp are attributable to the biological activity of its lipid A and its beneficial therapeutic effects, although the structure has not been determined.

To verify this, we are now trying to separate low molecular mass and high molecular mass components from original LPSp to analyze the difference in bioactivity depending on the polysaccharide chain length and to determine the structure of the polysaccharide moiety of LPSp. In conclusion, we determined the structure of the lipid A part of LPS from *P. agglomerans*. The results show that LPS from *P. agglomerans* is composed of at least two kinds of lipid A which are acylated differently: one is the same as those of *Salmonella typhimurium* or *E. coli* (hexa-acyl lipid A) [2,3] and the other is the same as that of *Salmonella minnesota* (hepta-acyl lipid A) [2].

References

[1] E.T. Rietschel, T. Kirikae, F. Ulrich, U. Schade, A.J. Ulmer, O. Holst, H. Brade, G. Schmidt, U. Mamat, H.-D. Grimmecke, S. Kusumoto, U. Zahringer, The chemical structure of bacterial endotoxin in relation to bioactivity, Immunobiology 187 (1993) 169–190.

[2] C. Galanos, O. Luderitz, M. Freudenberg, L. Brade, U. Schade, E.T. Rietschel, S. Kusumoto, T. Shiba, Biological activity of synthetic hepta acyl lipid A representing a component of *Salmonella minnesota* R595 lipid A, Eur. J. Biochem. 160 (1986) 55–59.

[3] M. Imoto, S. Kusumoto, T. Shiba, E.T. Rietschel, C. Galanos, O. Luderitz, Chemical structure of *Escherichia coli* lipid A, Tetrahedron Lett. 25 (1985) 907–908.

[4] T. Nishizawa, H. Inagawa, H. Oshima, T. Okutomi, D. Tsukioka, M. Iguchi, G.-I. Soma, D. Mizuno, Homeostasis as regulated by activated macrophage. I. Lipopolysaccharide (LPS) from wheat flour: isolation, purification and some biological activities, Chem. Pharm. Bull. 40 (1992) 479–483.

[5] D. Mizuno, D.-I. Soma, Oral or percutaneous administration of lipopolysaccharide of small molecular size may cure various intractable diseases: a new version of Coley's toxin, Mol. Biother. 4 (1992) 166–169.

[6] J. Kamei, Y. Iwamoto, T. Suzuki, M. Misawa, Y. Kasuya, H. Nagase, T. Okutomi, G.-I. Soma, D. Mizuno, Antinociceptive effect of lipopolysaccharide from *Pantoea agglomerans* on streptozotocin-induced diabetic mice, Eur. J. Pharmacol. 251 (1994) 95–98.

[7] T. Okutomi, T. Nishizawa, H. Inagawa, G.-I. Soma, M. Minami, D. Mizuno, Inhibition of morphine dependence by lipopolysaccharide from *Pantoea agglomerans*, Eur. Cytokine Netw. 3 (1992) 417–420.

[8] T. Suzuki, M. Funada, Y. Sugano, M. Misawa, T. Okutomi, G.-I. Soma, D. Mizuno, Effects of lipopolysaccharide from *Pantoea agglomerans* on the cocaine-induced place preference, Life Sci. 54 (1994) PL75–80.

[9] S. Goto, S. Sakai, J. Kera, Y. Suma, G.-I. Soma, S. Takeuchi, Intradermal administration of lipopolysaccharide in treatment of human cancer, Cancer Immunol. Immunother. 42 (1996) 255–261.

[10] N. Qureshi, R.J. Cotter, T. Takayama, Application of fast atom bombardment mass spectrometry and nuclear magnetic resonance on the structural analysis of purified lipid A component, J. Microbiol. Methods 5 (1986) 65–77.

[11] H.W. Wollenweber, K.W. Broady, O. Luderitz, E.T. Rietschel, The chemical structure of lipid A. Demonstration of amide-linked 3-acyloxyacyl residues in *Salmonella minnesota* Re lipopolysaccharide, Eur. J. Biochem. 124 (1982) 191–198.