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A novel cytokine-inducing glycolipid isolated from the lipoteichoic acid fraction of *Enterococcus hirae* ATCC 9790: A fundamental structure of the hydrophilic part

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Previously, we showed that quantitatively minor several glycolipids totaling only less than 5% of the lipoteichoic acid (LTA) fraction from *Enterococcus hirae* ATCC 9790 possessed cytokine-inducing activity, whereas the major component (over 90%) did not [Suda *et al.* (1995) *FEMS Immun Med Microbiol* 12:97–112]. The major inactive component was shown to have the chemical structure as was proposed for the LTA by Fischer [Hashimoto *et al.* (1997) *J Biochem* 121:779–86], suggesting that so-called LTA is not a cytokine-inducing component in the Gram-positive bacteria. In the present paper, the structure of the hydrophilic part of one of the cytokine-inducing glycolipid tentatively named GL4 is elucidated. GL4 was first subjected to hydrolysis with aqueous HF to give a polysaccharide and a mixture of low molecular weight products. The polysaccharide was composed mainly of highly branching mannan as concluded from NMR and MS analyses of its acetolysis products. The low molecular weight products consisted of phosphate and glycerol, suggesting the presence of a poly(glycerophosphate) structure in the original GL4. From these observations, the hydrophilic part of GL4 was shown to consist of mannose-rich polysaccharide and poly(glycerophosphate), the latter being bound to the former by a phosphodiester linkage.

Keywords: **acetolysis, IL-6, MALDI-TOF-MS, mannan, NMR, poly(glycerophosphate) structure**

Introduction

Lipoteichoic acid (LTA) is a macroamphiphile widely distributed on the cell surface of Gram-positive bacteria. The chemical structures of LTA from various bacterial species have been already proposed by the extensive study by Fischer [1]. In recent years, several research groups have described that LTA shows immunobiological activities similar to those of the lipopolysaccharide (LPS) of Gram-negative bacteria [2–5]. In the above biological studies, the glycolipid fraction extracted from bacterial cells by Fischer's procedure was used. Hence, the component with the structure proposed by him was automatically believed, without any reservation, to be responsible for the observed activities though no direct evidences were available. We previously reported fractionation of an LTA fraction (LTA-2) from *Enterococcus hirae* ATCC 9790 into several cytokine-inducing minor components and a major, but not cytokine-inducing, component [6]. The chemical composition analysis and the SDS-PAGE profiles of the former active components revealed that all of them are high-molecular weight glycolipids. The chemical structures of the active components were, however, not determined at that time because of their low contents. To elucidate their structures, isolation of larger amounts of the active components was inevitably required. In our previous work, a rapid and efficient large scale fractionation procedure was established using batch-wise and stepwise elution to give the cytokine-inducing minor fractions free from the inactive major components [7]. We also demonstrated that the structure of one of the inactive major components coincided with the structure proposed by Fischer [1] for an LTA (LTA-1) isolated from the same species of bacteria. The result unequivocally proved that the molecular species with the Fisher's LTA structure is not responsible for the cytokine-inducing activ-

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ity. Kusunoki *et al.* [8,9] also reported independently the occurrence and partial purification of a minor component, which induces cytokine production, from the LTA fraction of *Staphylococcus aureus.* Although their component has not been structurally characterized, their results are in good agreement with our findings.

In the present paper, we report the structural investigation of the hydrophilic part in a novel glycolipid (named GL4) which is one of the cytokine-inducing components in the LTA fraction isolated in our previous study.

Materials and methods

Isolation of cytokine-inducing components from bacterial cells

The cytokine-inducing fraction (QM-A) was prepared from *E. hirae* ATCC 9790 cells as described [7]. A total weight of 223 mg was separated from 922 g of dried bacterial cells.

Fractionation of QM-A on Octyl-Sepharose was performed according to the method of Suda *et al.* [6]. Briefly, a portion of QM-A (121 mg) was dissolved in 0.1 M acetate buffer (pH 4.5) containing 15% 1-propanol and applied to an Octyl-Sepharose CL-4B column (2.5 × 30 cm, Pharmacia LKB, Uppsala, Sweden) equilibrated with the same buffer. The column was eluted with a linear gradient of 1-propanol (15–60%). Based on the phosphorus contents, eluates were divided into five fractions (designated OS-1 to -5, Fig. 1), dialyzed, and lyophilized separately. By repeating the same chromatography, the following total amount of each fraction was obtained from QM-A (214 mg): OS-1 (19 mg), OS-2 (2.8 mg), OS-3 (8.3 mg), OS-4 (101 mg) and OS-5 (33 mg).

A portion of OS-4 (42.3 mg) was dissolved in 0.01 M acetate buffer (pH 4.5) containing 35% 1-propanol and applied to QMA-Mem Sep 1010 (PerSeptive Biosystems, Framingham, MA, USA) equilibrated with the same buffer. The membrane was eluted with a linear gradient of NaCl (0–1 M). Based on the hexose contents, two fractions, a low-anionic fraction (OS-4L) and a high-anionic fraction (OS-4H), were obtained, dialyzed, and lyophilized separately. By repeating similar separation once more, the total amount of OS- 4L and OS-4H weighed 11 and 42 mg from OS-4 (95 mg), respectively. As described in Results and discussion, the fraction OS-4L was considered to contain a novel glycolipid, named GL4.

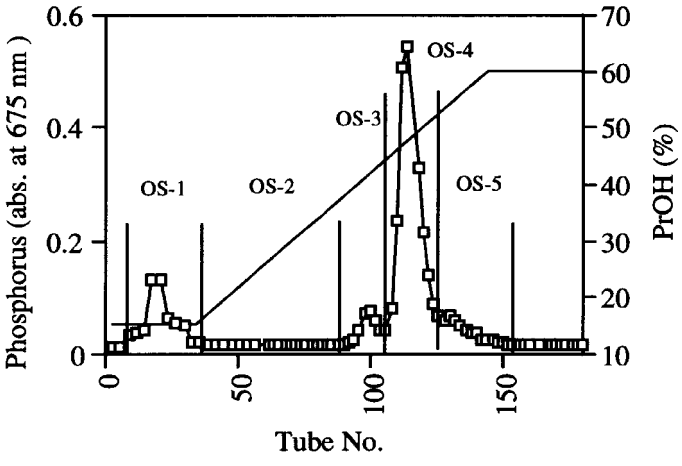

Figure 1. The elution profile of QM-A on Octyl-Sepharose CL-4B.

Analytical procedures

Phosphorus, fatty acids, carbohydrates, glycerol, and amino acids were analyzed by the method as described in our previous paper [6] and references cited therein. HPLC [7], determination of phosphomonoester [7], reducing sugar analysis [10], methylation analysis [11], and hexose analysis [12] were performed as described in the respective literatures.

TLC was performed on silica-gel plates (Merck silica gel 60 F_{254} No. 5715) using a solvent system chloroform-acetone (5/1, v/v). Spots on the plate were visualized by the use of iodine vapor.

NMR spectra

^{1}H NMR spectra were measured on a JMN-LA500 spectrometer (JEOL, Tokyo, Japan) at 500 MHz, a UNITY plus spectrometer (Varian, Palo Alto, CA, USA) at 600 MHz, or a UNITY INOVA spectrometer (Varian) at 750 MHz. ^{13}C NMR spectra were measured on a JMN-LA500 spectrometer at 126 MHz and a UNITY plus spectrometer at 151 MHz. The chemical shifts are expressed in δ values by using tetramethylsilane (δ 0) or water (δ 4.65) as the internal standards for ^{1}H spectra, and tetramethylsilane (δ 0) as the internal or benzene (δ 128) as the external standard for ^{13}C spectra.

Mass spectra

FAB-MS was obtained with an SX-102 mass spectrometer (JEOL). *m*-Nitrobenzyl alcohol was used as a matrix. MALDI-TOF-MS was obtained with a Kratos Kompact MALDI IV (Shimadzu, Kyoto, Japan) and a VOYAGER-ELITE-DE (PerSeptive Biosystems) mass spectrometer using 2,5-dihydroxybenzoic acid as a matrix. ESI-MS was obtained with Mariner (PerSeptive Biosystems) instrument.

Induction of IL-6 and *Limulus* assay

IL-6 induction in human peripheral whole-blood cell culture [13,14] and determination of its level using ELISA were performed as described [6]. *Limulus* activity of a sample was measured by means of Endospecy Test® (Seikagaku Corporation, Tokyo, Japan) using an LPS specimen from *Escherichia coli* O111:B4 (Sigma Co., St. Louis, MO, USA) as a reference standard as described [6].

Deacylation, hydrolysis and acetolysis

Deacylation of GL4 was performed as described [15].

Hydrolysis of GL4 with 47% aqueous HF and phase partition of the reaction mixture were performed according to the procedure of Fischer [1]. In brief, 3 mg of GL4 was hydrolyzed with 50 µl of 47% aqueous HF at 4°C for 24 h. The reaction mixture was dried *in vacuo* over sodium hydroxide. The residue was partitioned with a two-phase solvent system composed of chloroform, methanol, and water (2/1/3, v/v/v). The products extracted in the aqueous phase were fractionated on a Sephadex G-100 column (1.5 × 95 cm, Pharmacia). Based on the phosphorus and hexose contents, eluates were combined into three fractions (designated G100-I, -II and -III). Each fraction weighted 2.2, 0.5 and 0.3 mg, respectively.

Acetolysis was performed according to the method of Shibata [16] with a slight modification. G100-I (0.2 mg) was dissolved in 5 µl of formamide by sonication. After addition of 5 µl of pyridine and 5 µl of acetic anhydride, the mixture was heated at 40°C for 12 h. After addition of 0.5 ml of water, the reaction mixture was centrifuged at 1000 x g for 5 min. The precipitate was separated, washed twice with 0.5 ml of water, and dried *in vacuo.* The precipitate was dissolved in 25 µl of a mixture of acetic acid, acetic anhydride, and sulfuric acid (100/100/1, v/v/v) and heated at 40°C for 12 h. The reaction mixture was partitioned between 0.5 ml of water and 0.5 ml of chloroform. The chloroform phase was separated, washed five times with each 0.5 ml of water, and concentrated *in vacuo* to dryness with a rotary evaporator. The products were separated by TLC. Standard fully acetylated oligomannosides were prepared by acetolysis of commercially available mannan (from *Saccharomyces cerevisiae,* Sigma) followed by separation of the products in the same way.

Results and discussion

In our previous work [7], fractionation of the crude LTA was performed in two steps: (i) batch-wise elution on Octyl-Sepharose of the crude LTA to give two fractions (BOS40 and BOS60); (ii) stepwise elution on QMA-Mem Sep 1010 of each fraction to give a low-anionic, cytokine-inducing fraction (QM-A) and a high-anionic, non-cytokine-inducing fraction (QM-I). In the present study, further fractionation of QM-A was performed. Since no distinct differences in IL-6 inducing activity was observed between QM-A from BOS40 and QM-A from BOS60, the two fractions were combined and subjected to hydrophobic interaction column chromatography on Octyl-Sepharose CL-4B according to the method of Suda *et al.* [6] to give five fractions, OS-1 to OS-5 (Fig. 1). We focused on the major fraction among them, OS-4, which was again subjected to ion-exchange membrane chromatography to give low and high anionic fractions (OS-4L and OS-4H, Fig. 2). From the results of ESI-MS of its HF-hydrolysate (data not shown), OS-4H was characterized as identical with the contaminating high anionic non-cytokine-inducing component (QM-I).

OS-4L gave no definite peak on direct gel permeation HPLC presumably because of mutual aggregation owing to its amphiphilic nature. A small portion of OS-4L was thus subjected to alkaline hydrolysis for deacylation. The deacylated product was eluted as a broad but single peak in this gel permeation HPLC. Its molecular weight at the peak top was deduced to be 4×10^3 by using standard heparins or 1×10^4 by using dextrans, respectively. OS-4L was then analyzed by MALDI-TOF-MS. Several distinct peaks in the range of m/z 6.5–8.5 $\times 10^3$ (the major peaks appeared at intervals of about m/z 160 and some weak peaks were observed near of them) were observed in the linear and positive mode (Fig. 3). These ions appeared just around the average of the mass ranges deduced by the above HPLC. The presence of multiple ion peaks are explained by the occurrence of congeners which contain different numbers of repeating units or carbohydrate components and/or diversity of fatty acids. In view of these observation and the multi-step chromatographic procedures of different principles employed for its separation, the fraction OS-4L was considered to contain a single kind of glycolipid which was tentatively named GL4 hereafter.

The IL-6 inducing activity and *Limulus* activity of the starting QM-A, the separated GL4 and OS-4H are summarized in Table 1. The IL-6 inducing activity of GL4 was 30 times higher than that of OS-4H, though both of them exhibit identical level of *Limulus* activity. It should be emphasized here that IL-6 inducing potency of the former GL4 was more than 150 times as high as the value expected for the identical amount of LPS which would give the same level of *Limulus* response as observed with GL4. This fact indicated that the IL-6 inducing activity of GL4 is inherent in itself: the presence of contaminating LPS is unequivocally excluded.

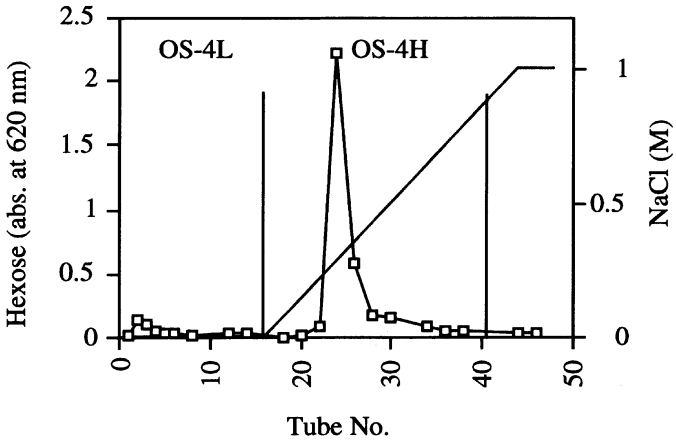

Figure 2. The elution profile of OS-4 on QMA-Mem Sep 1010.

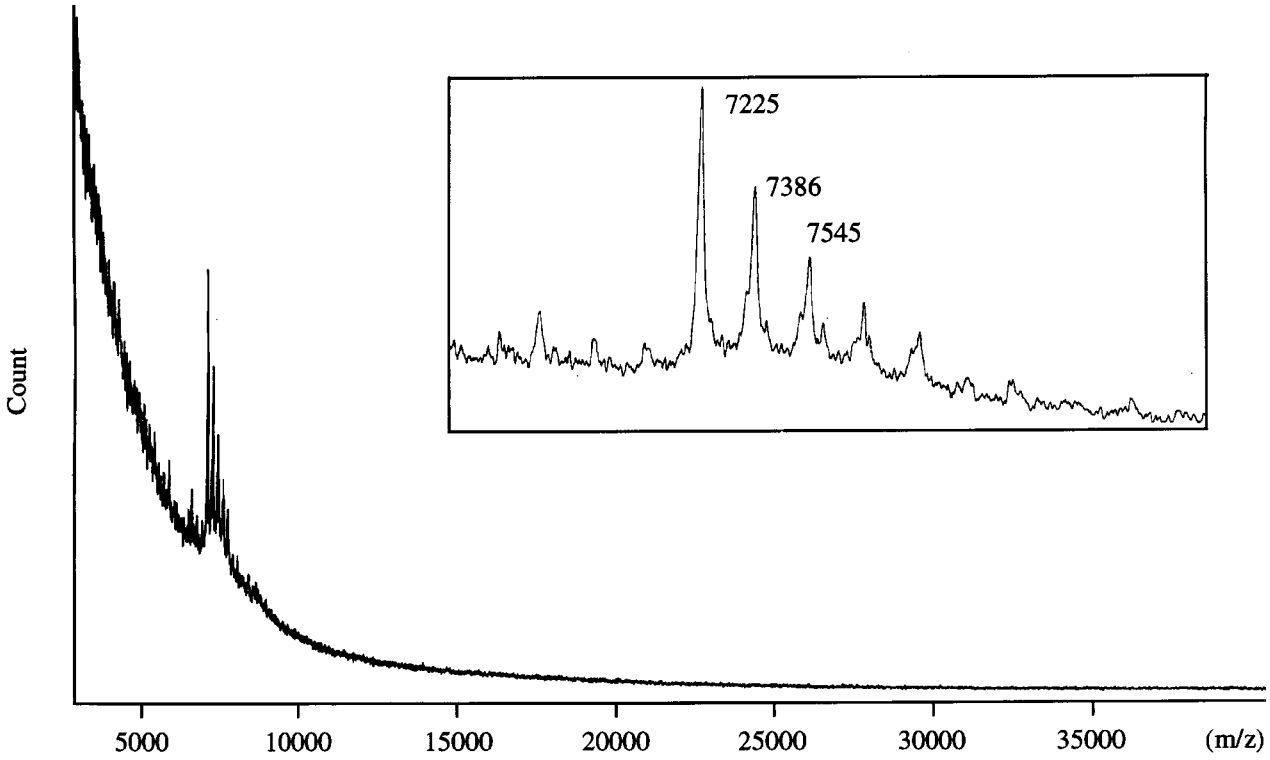

Figure 3. MALDI-TOF mass spectrum of GL4 with VOYAGER-ELITE-DE in the linear and positive mode. 2,5-Dihydroxybenzoic acid was used as a matrix. The peak at *ca.* 4000 arises from the matrix.

Limulus activity observed for GL4 is also assumed to be inherent in itself but not due to LPS. This assumption would be rational since *Limulus* activity has already been proven not exclusively specific to LPS. Some acidic phospholipids were found to be definitely active in *Limulus* gelation test [17].

The chemical compositions of GL4 are summarized in Table 2. GL4 consists of phosphate, carbohydrates, fatty acids, and glycerol. Since no free phosphate was liberated by direct alkaline phosphatase digestion of GL4, the presence of phosphomonoesters was excluded. Carbohydrates consist of mannose and glucose (molar ratio, 5.3:1.0). On methylation analysis (Table 3), the mannose residues were characterized as non-substituted, 2-*O*-substituted, and 2,6-di-*O*-substituted forms. 3-*O*-Substituted and 6-*O*-substituted mannose residues were also detected but in much lower amounts. Glucose was detected only as a non-substituted form: no occurrence of substituted glucose was observed at all. No reducing sugar was detected. Fatty acids which were mainly hexadecanoic and octadecenoic acids were linked *via* ester linkages, because they were liberated by alkaline hydrolysis.

Table 1. IL-6 inducing activity and *Limulus* activity

	Combined QM-A	*GL4*	*OS-4H*
IL-6 inducing activity ($\times 10^{-4}$ g)[a]	80	300	10
Limulus activity ($\times 10^{-4}$ g)[a]	2.1	1.9	1.9

[a]The potencies of IL-6 inducing and *Limulus* activity are expressed in terms of the amount of LPS (from *E. coli* O111:B4, Sigma) which induces the same magnitude of responses observed with 1 g of each test specimen.

Table 2. Chemical composition of GL4

	μmol / mg	*Molar ratio*[a]
Phosphate	0.61	1.0
Carbohydrates	3.44	
Man	2.89	4.8
Glc	0.55	0.90
Fatty acids	0.36	
14:0	0.02	0.02
16:0	0.14	0.24
16:1	0.03	0.05
18:0	0.03	0.05
18:1	0.14	0.24
Glycerol	0.77	1.3

[a]Molar ratios are calculated by assuming the presence of one mole of phosphate.

Table 3. Methylation analysis of GL4 and G100-I

	Molar ratio[a]	
	GL4	*G100-I*
O-substituted mannose		
non-	1.00	1.00
2-	0.86	0.84
3-	b	b
6-	b	b
2,6-di-	1.28	1.38
non-substituted glucose	0.21	0.12

[a]Molar ratio was estimated from the peak areas in GC analysis.
[b]Quantitative analysis failed because of overlapping of unknown minor peaks.

In our previous fractionation of LTA of the same bacteria [6], none of the separated active fractions contained mannose. This discrepancy may be explained as follows. In the previous work, only the more hydrophobic part of LTA (LTA-2) was used as the starting material because Tsutsui *et al.* [18] reported that the corresponding fraction possessed stronger cytokine-inducing and antitumor activities than those of the less hydrophobic part (LTA-1) in a murine assay system. Later we re-examined the activity of the LTA-1 fraction using human peripheral whole blood cells and found that LTA-1 also showed IL-6 inducing activity comparable to that of LTA-2. In the present work, we first fractionated both LTA fractions, BOS40 and 60 (correspond to LTA-1 and -2, respectively), separately but then combined two active fractions (QM-A) before further fractionation. Thus, the GL4 is assumed to originate mainly from the less hydrophobic BOS40 (LTA-1) fraction.

The structure of GL4 was first analyzed by NMR. The ^{1}H NMR spectrum of GL4 is shown in Figure 4a. Because of

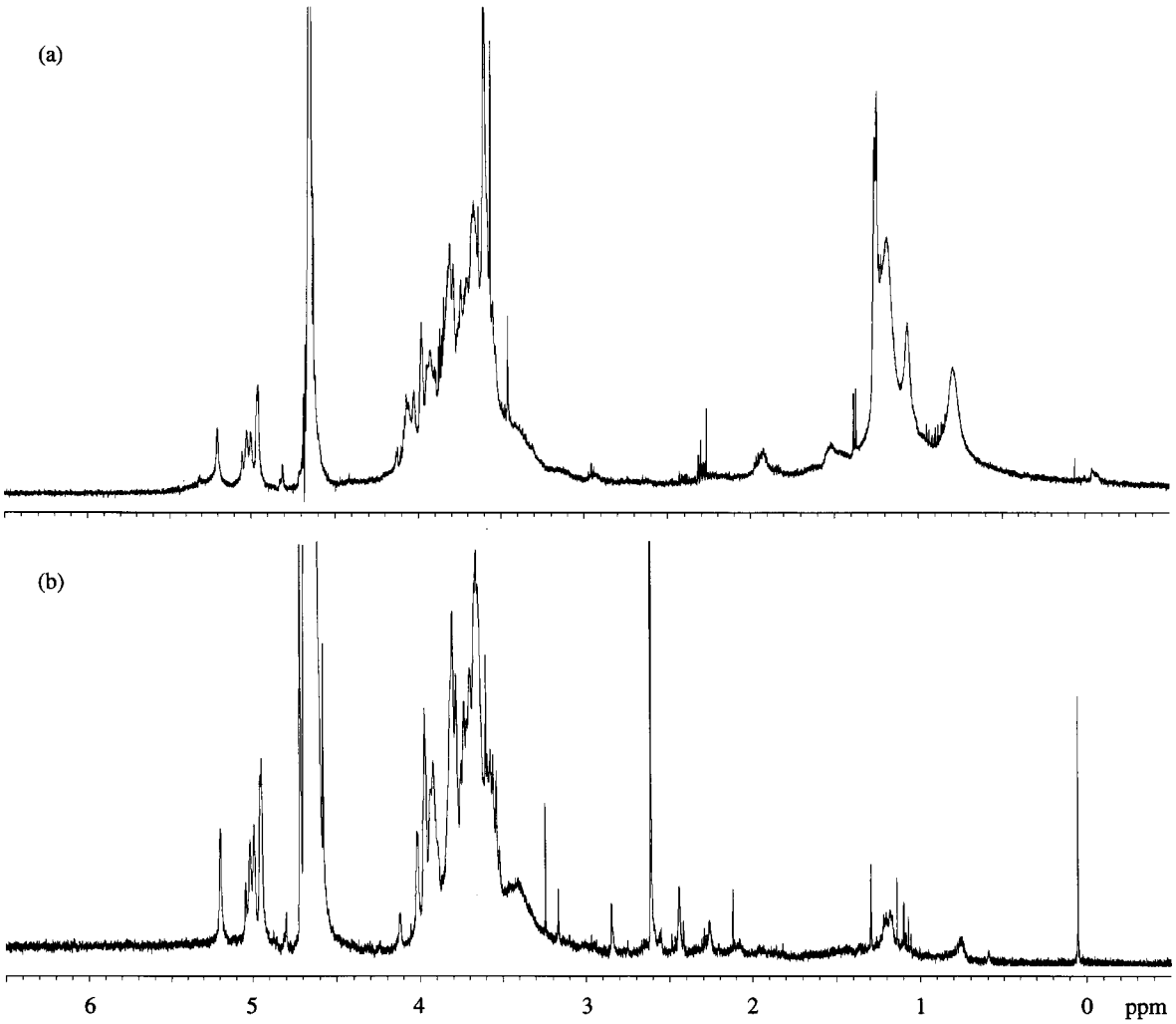

Figure 4. ^{1}H NMR spectrum of GL4 (a) and G100-I (b). The spectrum was measured in D_2O at 303K.

the broad and overlapping signals due to the high molecular weight and micellar aggregation, only their partial assignment was possible. To reduce the molecular size, GL4 was subjected to HF hydrolysis to cleave off the phosphodiester linkages. The ^{1}H NMR spectrum of the products extracted into the aqueous phase from the HF-hydrolysate indicated that most of the fatty acids were removed, suggesting that the lipophilic part containing fatty acids was bound to the hydrophilic region of GL4 via phosphodiester linkages (data not shown). The hydrophilic products were separated by gel filtration chromatography on Sephadex G-100 into three fractions, G100-I, -II, and -III (Fig. 5).

The chemical composition analysis of G100-I showed that it contains mannose (1.1 μmol/mg) and glucose (0.18 μmol/mg), but no phosphate or glycerol. As shown in Figure 4b, the ^{1}H NMR signals of the anomeric protons (δ 4.7–5.2) of the carbohydrate moieties in G100-I were almost identical to those of GL4. The carbohydrate part which represents the polysaccharide core of GL4 was thus concluded not to have changed by the HF hydrolysis. These results proved that the glucose in the GL4 originated not from contaminating LTA which have oligo-glucosyl-glycerol, but from GL4 itself.

From the result of the methylation analysis (Table 3), the main mannose residues in G100-I are of non-substituted, 2-*O*-substituted, and 2,6-di-*O*-substituted forms, suggesting the branching mannan containing 1–2 and 1–6 linkages. To analyze the structure of the polysaccharide in more details, G100-1 was subjected to acetolysis according to the method of Shibata *et al.* [16]. A commercially available mannan from *S. cerevisiae* was first treated by this method to evaluate the selectivity of the preferential cleavage of 1–6 linkages and to prepare standard oligosaccharides of mannose. Fully acetylated Man, Man(α1-2)Man, Man(α1-2)Man(α1-2)Man, and Man(α1-3)Man(α1-2)Man(α1-2)Man were obtained in good yields as described [19]. In all the oligomannosides isolated, no Man(1-6)Man linkage was retained: the highly selective cleavage of Man(1-6)Man linkage was confirmed under the conditions employed for the acetolysis. Then G100-I was subjected to acetolysis under the same conditions and the products were analyzed by MALDI-TOF-MS (Fig. 6). In the reflectron and positive mode, distinct ion peaks at m/z 700, 988, 1276, and 1564 were observed. These were assigned to the sodium adduct ions of fully acetylated Hex_n (n = 2 − 5). The products were then separated by preparative TLC into four major products, designated M1 (R_f 0.77), M2 (R_f 0.69), M3, (R_f 0.60) and M4 (R_f 0.48). The products containing only mannose were characterized on the basis of their FAB-MS and ^{1}H-NMR data as far as their limited amounts allowed (Table 4).

M1 was characterized as fully acetylated α-mannose by ^{1}H NMR spectra. In the positive mode FAB-MS analysis of M2, an ion peak at m/z 701 was observed, corresponding to the sodium adduct ion of fully acetylated Man_2. No downfield shifts due to acetylation of Man^I H-2 and Man^{II} H-1 in M2 were observed in the ^{1}H NMR spectra, indicating Man^{II}(1-2)Man^I linkage. The 1–2 glyxosidic linkage was confirmed by one dimensional NOE spectra, in which an interresidual correlation between Man^{II} H-1 and Man^I H-2 was observed. An ion peak at m/z 989 observed for M3 corresponds to the sodium adduct ion of fully acetylated Man_3. No downfield shifts of Man^I H-2, Man^{II} H-2, Man^{II} H-1, and Man^{III} H-1, and interresidual correlations between Man^{II} H-1 and Man^I H-2, and Man^{III} H-1 and Man^{II} H-2 in NOESY spectra (Fig. 7) showed the Man^{III}(1-2)Man^{II}(1-2)Man^I sequence. An ion peak at m/z 1277 found for M4 coincides with the mass number of the sodium adduct of fully acetylated Man_4. No downfield shifts of three Man H-1, two Man H-2, and one Man H-3 were

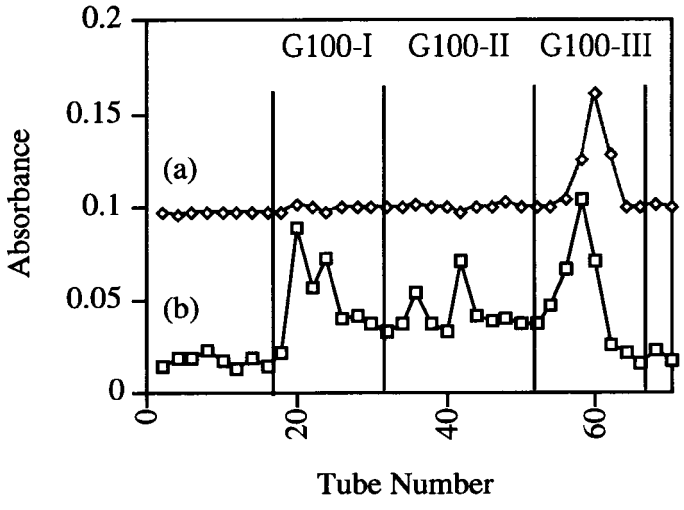

Figure 5. The elution profile on Sephadex G-100 of the products extracted in the aqueous phase from the HF-hydrolysate of GL4. The fractions were analyzed for (a) phosphorus (absorbance at 675 nm) and (b) hexose (absorbance at 620 nm).

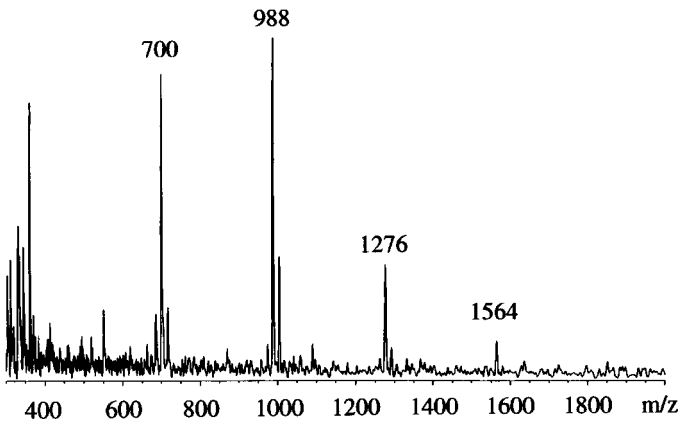

Figure 6. MALDI-TOF-MS analysis of acetolysis products from G100-I with KOMPACT MALDI IV in the refrectron and positive mode. 2,5-Dihydroxybenzoic acid was used as a matrix.

Table 4. ^{1}H NMR data for the acetolysis products from G100-I. The spectra of M2 and M3 were measured at 500 MHz in $CDCl_3$ at 303K and those of M4 at 600 MHz or 750 MHz in $CDCl_3$ at 303K. The assignments were established by analysis of ^{1}H one-dimensional spectra, one-dimensional NOE spectra, and two-dimensional methods, COSY and NOESY. Mannose residues are numbered from the reducing to the non-reducing termini.

	Chemical shift δ	*(Coupling constants) (Hz)*	
Proton	*M2*	*M3*	*M4*
Man^{I} H-1	6.23 (2.1)	6.23 (1.8)	6.25 (1.9)
Man^{I} H-2	4.03 (2.3, 3.2)	4.06	4.05
Man^{I} H-3	5.28 (3.1, 10.2)	5.30	5.32
Man^{I} H-4	5.42 (10.3, 10.1)	nd	nd
Man^{I} H-5	3.99	nd	nd
Man^{I} H-6	4.22 (4.1, 12.4)	nd	nd
	4.12 (2.3, 12.1)		
Man^{II} H-1	4.94 (2.1)	5.11 (2.1)	5.11 (1.6)
Man^{II} H-2	5.25	4.11	4.11
Man^{II} H-3	5.39 (3.2, 10.0)	5.29	nd
Man^{II} H-4	5.25	nd	nd
Man^{II} H-5	4.17	nd	nd
Man^{II} H-6	nd	nd	nd
Man^{III} H-1		4.94 (1.9)	4.94 (1.9)
Man^{III} H-2		5.27	5.23
Man^{III} H-3		nd	4.14
Man^{III} H-4		nd	nd
Man^{III} H-5		nd	nd
Man^{III} H-6		nd	nd
Man^{IV} H-1			5.04 (1.9)
Man^{IV} H-2			5.07 (1.9, 3.6)
Man^{IV} H-3			nd
Man^{IV} H-4			nd
Man^{IV} H-5			nd
Man^{IV} H-6			nd
			nd

nd: not determined because of signal overlapping.

observed, indicating two Man(1-2)Man linkages and a Man(1-3)Man linkage. The complete sequence of M4, however, was not determined directly because the measurement of NOE spectra was not successful owing to its low quantity.

Further structural assignment including anomeric configurations of the acetolysis products from G100-I was completed by comparison with standard fully acetylated oligosaccharides obtained from acetolysis of *Saccharomyces* mannan, whose structures were unequivocally determined by complete assignment of their NMR signals using DEPT, HMQC, and HMBC techniques (Table 5). M2, M3, and M4 were proved to be identical with fully acetylated Man(α1-2)Man, Man(α1-2)Man(α1-2)Man, and Man(α1-3)Man(α1-2)Man(α1-2)Man, respectively.

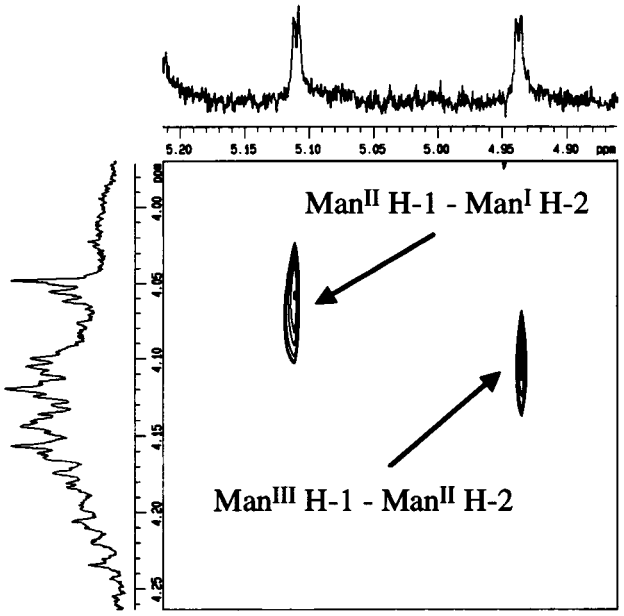

Figure 7. A part of NOESY spectrum of M3. The spectrum was measured in $CDCl_3$ at 303K. The mixing time was 650 ms.

Since the present acetolysis reaction cleaves only Man(1-6)Man linkages, the oligomannosides (M1, M2, M3, and M4) are assumed to be connected to each other via Man(1-6)Man linkages. The molar ratio of each subunit (x:y:z:w = 1:4:7:1) was calculated from the peak area of H-1 in ^{1}H NMR of G100-I (Fig. 8). In the case of overlapping H-1 signals (δ around 4.96–4.99), their intensities were estimated from the intensities of respective H-2 signals. Correlations of individual mannose units were assigned according to the reference [16]. Although the location of the glucose substitution in G100-I could not be determined, it may be considered that glucose moieties are attached to the mannan core polysaccharide via Glc-Man(1-6)- linkages.

The chemical composition and the ^{1}H NMR spectrum of G100-II were almost identical to those of G100-I, suggesting a common structural principle except for the molecular weight. G100-III which was eluted at the bed volume of the Sephadex G-100 column is expected to consist of a mixture of low molecular weight (less than 10^3) compounds. The chemical composition analysis of G100-III showed phosphate (1.2 μmol/mg), glycerol (1.2 μmol/mg), and a trace amount of glucose (0.1 μmol/mg), but no mannose. These data suggest the presence of a glycerophosphate structure in GL4.

The ratio of the methylated mannose derivatives obtained by methylation analysis of GL4 was almost identical to that of G100-I (Table 3). This fact suggests that the number of the glycerophosphate moieties directly attached to the polysaccharide core is very limited. The phosphate and glycerol liberated on HF hydrolysis are thus concluded to be present originally in a form of a poly(glycerophos-

Table 5. ^{1}H NMR data for the standard fully acetylated oligomannoses, fully acetylated Man(α1-2)Man, Man(α1-2)Man(α1-2)Man, and Man(α1-3)Man(α1-2)Man(α1-2)Man. The spectra were measured at 500 MHz in $CDCl_3$ at 303K. The assignments were established by analysis of ^{1}H and ^{13}C one-dimensional spectra, ^{13}C DEPT spectra, and following two-dimensional methods: COSY, NOESY, HMQC, and HMBC. Mannose residues are numbered from the reducing to the non-reducing termini.

	Chemical shift δ	*(Coupling constants) (Hz)*	
Proton	*Man(α1-2)Man*	*Man(α1-2)Man(α1-2)Man*	*Man(α1-3)Man(α1-2)Man(α1-2)Man*
Man^{I} H-1	6.22 (2.3)	6.22 (2.1)	6.23 (2.3)
Man^{I} H-2	4.03 (2.3, 3.0)	4.05 (2.7)	4.04 (2.7, 5.5)
Man^{I} H-3	5.28 (3.2, 10.1)	5.30	5.29
Man^{I} H-4	5.41 (10.3, 10.0)	5.37 (9.6, 9.6)	5.38 (9.8, 9.9)
Man^{I} H-5	3.99 (2.3, 3.7, 10.0)	4.00	4.00
Man^{I} H-6	4.22 (3.9, 12.6)	4.22 (4.2, 12.6)	4.22 (4.0, 12.4)
	4.11 (2.4, 12.4)	4.10	4.10
Man^{II} H-1	4.93 (1.8)	5.11 (2.1)	5.09 (1.8)
Man^{II} H-2	5.25	4.11	4.09
Man^{II} H-3	5.39 (3.4, 10.0)	5.30	5.30
Man^{II} H-4	5.25	5.30	5.21 (9.9, 10.0)
Man^{II} H-5	4.17	4.10	4.11
Man^{II} H-6	nd	nd	4.12
Man^{III} H-1		4.92 (1.8)	4.92 (1.8)
Man^{III} H-2		5.25 (1.9, 3.5)	5.22 (1.6, 3.8)
Man^{III} H-3		5.36 (10.1)	4.15
Man^{III} H-4		5.26 (10.0, 10.1)	5.28 (9.6, 9.7)
Man^{III} H-5		4.10	4.15
Man^{III} H-6		nd	4.18
Man^{IV} H-1			5.01 (1.6)
Man^{IV} H-2			5.05 (1.8, 3.5)
Man^{IV} H-3			5.19 (3.4, 10.0)
Man^{IV} H-4			5.31 (9.9, 10.0)
Man^{IV} H-5			4.00
Man^{IV} H-6			4.29 (4.0, 12.3)
			4.03

nd: not determined because of signal overlapping.

phate) structure which is linked at certain limited positions of the polysaccharide.

From all the data presented above, hydrophilic region of GL4 is concluded to consist of a mannose-rich highly branching polysaccharide core linked with poly(glycerophosphate) *via* phosphodiester linkage (Fig. 9).

Garner *et al.* [20] reported that mannan from the cell wall of *Candida albicans* stimulates the production of TNF-α by macrophage. Chatterjee *et al.* [21] and Suda *et al.* [22] reported that mannose containing glycolipids from several mycobacteria induce IL-6 and TNF-α. These observations show that the oligo- or polymannoside moiety may play an important role in stimulation of cytokine production. By contrast, we found that *S. cerevisiae* mannan whose structure is close to that of the mannose-rich polysaccharide core in GL4 was inactive for IL-6 induction in a human peripheral whole-blood test system. The hydrophilic products, from which G100-I, a mannan core substituted by glucose residues, was isolated, of HF-hydrolysis of GL4 were inactive either (data not shown). These facts suggest that not only the mannose moieties containing glucoses, but also other components, such as the lipophilic moiety and/or phosphates, are important for the activity. The hydrophobic products from HF-hydrolysis of GL4 are being characterized separately. Their structures and their mode of linkage to the hydrophilic part will be described soon elsewhere.

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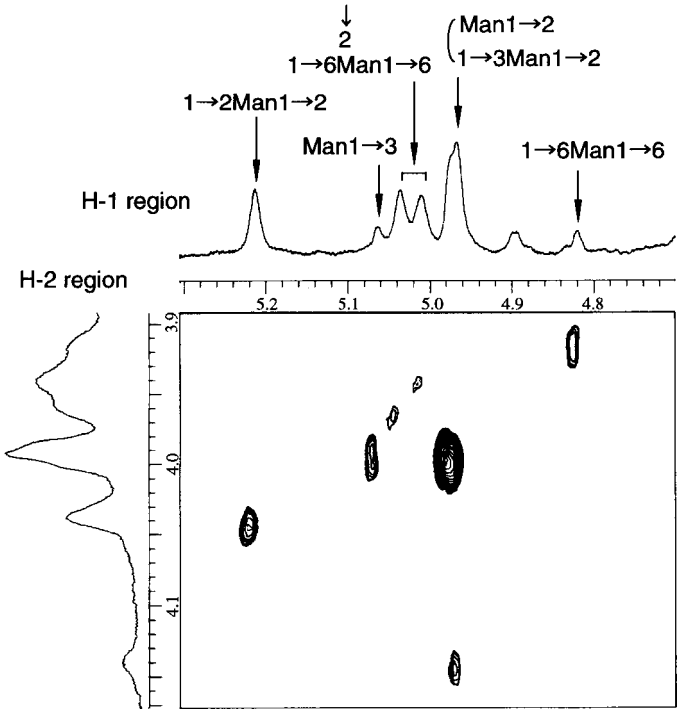

Figure 8. A part of COSY spectrum of G100-I.

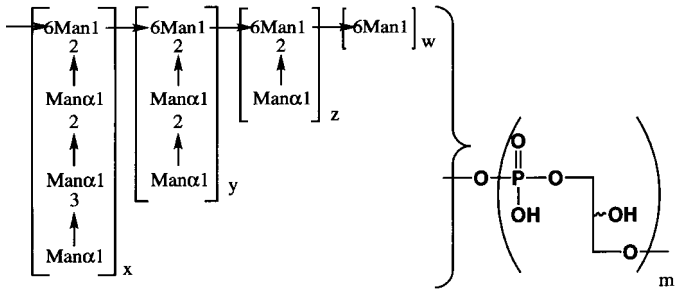

Figure 9. A fundamental structure proposed for the hydrophilic part of GL4 from the LTA fraction of *E. hirae* ATCC 9790. x:y:z:w = 1:4:7:1. The sequence of each substructure is interchangeable.

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