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Structural Feature of the Major but Not Cytokine-Inducing Molecular Species of Lipoteichoic Acid[1]

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Previously, lipoteichoic acid (LTA) of *Enterococcus hirae* was found to exhibit definite cytokine-inducing activity but synthetic specimens which share the fundamental structural principles proposed for LTA had no corresponding activity. We also showed recently that several minor components totally less than 5% of the LTA fraction from *E. hirae* ATCC 9790 possessed the activity, whereas the major component (over 90%) did not [Suda, Y., Tochio, H., Kawano, K., Takada, H., Yoshida, T., Kotani, S., and Kusumoto, S. (1995) *FEMS Immun. Med. Microbiol.* 12, 97-112]. In the present study, the structure of the major component of LTA was studied in an attempt to elucidate the reason for the lack of the activity in the synthetic compounds. The major component of the LTA was first digested by hydrofluoric acid hydrolysis to cleave phosphodiester linkages present. The hydrolysis products were separated and characterized by means of NMR and MS. The linkage positions of the original phosphodiesters were determined from the NMR spectra of an alkali-treated product without hydrofluoric acid degradation. The compound was proved to consist of 1,3-linked poly(glycerophosphate) and a lipid anchor, Glc(α1-2)Glc(α1-3)acyl$_2$Gro, the former being linked to the 6-position of the distal glucose of the latter. The 2-position of the glycerol residues in the glycerophosphate part were substituted by oligoglucose esterified partially with alanine. The gross structure elucidated here thus coincides with the previous conclusion described by Fischer [Fischer, W. (1990) in *Glycolipids, Phosphoglycolipids and Sulfoglycolipids* (Kates, M., ed.) pp. 123-234, Plenum Press, New York]. Thus, the molecular species with this so-called "LTA structure" is not responsible for the cytokine-inducing activity.

Key words: glycolipid, lipoteichoic acid, mass spectrometry, NMR, structural analysis.

It has been reported that lipoteichoic acid (LTA) is a macroamphiphile widely distributed on the cell surface of Gram-positive bacteria, like lipopolysaccharide (LPS) of Gram-negative bacteria. The structures of LTAs from enterococcal and streptococcal species were proposed by Fischer (*1*). According to him, LTA is generally composed of a glycolipid anchor and a 1,3-linked poly(glycerophosphate) chain with substitution by oligoglucose or D-alanine at the 2-positions of the glycerols. The biological activities of these LTAs, such as cytokine [IL-1, IL-6, and TNF-α] inducing and antitumor activities, were reported by Yamamoto *et al.* (*2, 3*), Usami *et al.* (*4, 5*), Tsutsui *et al.* (*6*), and Bhakdi *et al.* (*7*), and reviewed by Kotani (*8*). To find the essential structure in the LTA responsible for cytokine-inducing activity, Fukase *et al.* (*9, 10*) synthesized the fundamental structures proposed for LTAs. Surprisingly, however, neither cytokine-inducing nor antitumor activity was observed in the synthetic compounds (*11*). The structure of the synthetic analogues had several obvious differences from those proposed for natural LTA: (i) the glycolipid anchor in natural LTA contains unsaturated fatty acids, whereas the synthetic ones contain only saturated acids; (ii) the poly(glycerophosphate) in natural LTA was assumed to have between 9 and 40 repeating units (*12*), whereas that of the synthetic ones contains only 4 units; (iii) the synthetic poly(glycerophosphate) part had no substituents in contrast to the high glucosyl and appreciable alanyl substitution described for natural LTA.

The lack of cytokine-inducing activity in the synthetic compounds may thus be attributable to either of the

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Abbreviations: acyl$_2$Gro, 1,2-di-*O*-acylglycerol; COSY, correlation spectroscopy; DEPT, distortionless enhancement by polarization transfer; DQF, double quantum filter; ESI-MS, electrospray ionization mass spectrometry; FAB-MS, fast atom bombardment mass spectrometry; Glc, glucose; Glc$_n$Gro, oligoglucosylglycerol; Gro, glycerol; HMBC, heteronuclear multiple bond connectivity; HMQC, homonuclear multi quantum coherence; IL, interleukin; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; TNF, tumor necrosis factor; TOCSY, total correlation spectroscopy.

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following two possibilities: (i) some or all of the above structural features are essential for the biological activities of LTA; (ii) unknown components present in LTA are responsible for the observed activity. We have recently succeeded in separating LTA from *Enterococcus hirae* ATCC 9790 into small amounts of biologically active components and an inactive major component which amounts to over 90 wt% (*13*); this favors the latter possibility rather than the former. To evaluate the former possibility, we analyzed, in the present study, the overall chemical structure of the biologically inactive major component of LTA, referring to the structure previously proposed by Fischer (*1*).

MATERIALS AND METHODS

Bacterial Cells—*E. hirae* ATCC 9790 was grown in 150-liter batches with stirring at 37°C for 6 h. The growth medium (1 liter) contained 10 g glucose, 15 g trypticase, 4 g tryptose, 4 g yeast extract, 2 g K_2HPO_4, 5 g KH_2PO_4, 2 g Na_2CO_3, and 2 g NaCl. The bacteria were harvested by centrifugation and stored in acetone at 4°C until use.

NMR Spectroscopy—Proton and ^{13}C NMR spectra were measured on a JMN-LA500 spectrometer (JEOL, Tokyo) at 500 and 126 MHz, respectively. The chemical shifts are expressed in δ values by using tetramethylsilane (δ 0) or water (δ 4.65) as the internal standard for ^1H spectra, and tetramethylsilane (δ 0) as the internal or benzene (δ 128) as the external standard for ^{13}C spectra.

Mass Spectrometry—FAB-MS were obtained with an SX-102 mass spectrometer (JEOL). Glycerol or *m*-nitrobenzyl alcohol was used as a matrix. ESI-MS were obtained with an API III plus mass spectrometer (PE SCIEX, Thornhill, Ontario, Canada).

Fractionation of Crude LTA by Octyl-Sepharose—Delipidation of the bacteria, hot phenol/water extraction, and DNase-RNase digestion of the extract were performed as described (*13*). The digested extract was dialyzed, concentrated, and lyophilized to give a crude LTA preparation. The yield was 2-3% based on the dry weight of the delipidated cells. A solution of the crude LTA (1 g) in 200 ml of 0.1 M acetate buffer (pH 4.5) containing 15% 1-propanol was added to 400 ml of Octyl-Sepharose (Pharmacia LKB, Uppsala, Sweden) equilibrated with the same buffer. The mixture was allowed to stand at 4°C for 30 min and filtered. The Octyl-Sepharose was again suspended in 250 ml of the same buffer, allowed to stand at 4°C for 30 min and filtered. This procedure was repeated 4 more times. The recovered Octyl-Sepharose was treated similarly with the acetate buffer containing 40% 1-propanol (4 times) and then with that containing 60% 1-propanol (6 times). The filtrates obtained were separately combined, concentrated *in vacuo* to about 100 ml, dialyzed, again concentrated *in vacuo*, and lyophilized to give three fractions, which were designated BOS15 (Batch-wise Octyl-Sepharose eluate with a buffer containing 15% 1-propanol), BOS40, and BOS60, respectively. The values of recovery of the respective fractions were *ca.* 30, 45, and 10% from the crude LTA.

Further Separation of the Major Octyl-Sepharose Fraction (BOS40)—A portion of BOS40 (50 mg) was dissolved in 6 ml of 0.01 M acetate buffer (pH 4.5) containing 35% 1-propanol and applied to an ion-exchange membrane, QMA-Mem Sep 1010 (PerSeptive Biosystems, Framingham, MA, USA), equilibrated with the same buffer. The membrane was washed with 100 ml of the same buffer at a flow rate of 3.3 ml/min. The eluate was concentrated *in vacuo* to about 40 ml with a rotary evaporator. The materials bound to the membrane were then eluted with 100 ml of the buffer containing 35% 1-propanol and 1 M NaCl. The eluate was concentrated *in vacuo* to about 40 ml with a rotary evaporator. Both eluates were dialyzed separately, concentrated, and lyophilized to give two fractions, QM-A and QM-I. Similar procedures were repeated several times and the recoveries of the two fractions based on BOS40 were in the ranges of 2-3 and 80-90%, respectively.

Analytical Procedures—Phosphorus, glycerol, glucose, fatty acids, and alanine were analyzed as described in the previous paper (*13*) and references cited therein.

TLC was performed on silica-gel plates (Merck Keiselgel 60 F_{254} Art. 5715). The following solvent systems were used: A, chloroform/methanol/water (65 : 25 : 4, v/v/v); B, chloroform/methanol/ethyl acetate (100 : 4 : 6, v/v/v). Spots on the plates were visualized by the use of iodine vapor, water spray, or an anisaldehyde-sulfuric acid reagent.

HPLC was carried out on an LC-6AC liquid chromatograph (Shimadzu, Tokyo) equipped with an RID-6A refractive index detector and a C-R7A Chromatopac. Gel permeation chromatography was performed on an Asahipak GFA-30 column (500 mm × 7.5 mm ID, Asahi Kasei, Kanagawa) using 0.01 M phosphate buffer (pH 7.0) containing 0.5 M NaCl as an eluent at a flow rate of 0.8 ml/min. Several heparin standards (molecular weights: 1.8×10^4, 9.8×10^3, 4.4×10^3, 2.4×10^3, and 1.2×10^3) and dextran standards (1.7×10^4, 1.15×10^4, and 9.4×10^3) were used to make calibration curves for the determination of the molecular weight.

Determination of Phosphomonoester in the High-Anionic Major Component (QM-I)—QM-I (1.5 mg) was dissolved in 1.5 ml of an alkaline phosphatase solution (from *Escherichia coli*, Wako Pure Chemicals, Osaka, 5 units/ml in 0.04 M ammonium carbonate). The mixture was incubated at 37°C for 24 h. The amount of free phosphate in the reaction mixture was then determined as described above.

Hydrolysis of QM-I—Hydrolysis of QM-I with 47% hydrofluoric acid and phase partition of the reaction products were performed according to the procedure of Fischer (*1*). In brief, 10 mg of QM-I was hydrolyzed with 50 μl of hydrofluoric acid 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 in the aqueous phase were treated with alkaline phosphatase at 37°C for 24 h in 0.04 M ammonium carbonate. After lyophilization, the mixture was peracetylated with acetic anhydride/pyridine (1 : 1, v/v) at room temperature for 24 h. After evaporation *in vacuo*, the peracetylated products were extracted with chloroform and separated by preparative TLC using solvent system B.

Induction and Determination of the Level of IL-6—IL-6 induction in human peripheral whole-blood cell culture (*14, 15*) and determination of its level using ELISA were performed as described (*13*).

Deacylation of QM-I—Deacylation of QM-I was per-

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formed according to the procedure of Kochanowski *et al.* (*16*): hydrolysis with 0.2 M sodium hydroxide at 37°C for 2 h, followed by removal of the fatty acids liberated, and desalting by dialysis.

Miscellaneous—We took care to avoid contamination of test materials and instruments with extraneous bacterial endotoxins. For example, endotoxin-free water purchased from Otsuka (Tokyo) or prepared by a combination of Toray Pure LV-308 (Toray, Tokyo) and GSL-200 (Advantec, Tokyo) was used throughout the study.

RESULTS AND DISCUSSION

In our previous work, fractionation of the crude LTA was performed by gel filtration chromatography followed by hydrophobic interaction chromatography on Octyl-Sepharose with a linear gradient of 1-propanol to give three fractions; nucleic acid containing fraction (NA), LTA-1 (recovery: 30-50%), and LTA-2 (recovery: 7-14%). This method, however, was not practical for large-scale fractionation because of a flow rate limitation. Thus, in the present study, a batch-wise method of fractionation on Octyl-Sepharose was employed to achieve rapid and efficient separation. The 1-propanol concentrations selected for the present elution correspond to those at the peak centers of the three individual fractions in our previous chromatography with a linear gradient. Three fractions, BOS15, BOS40, and BOS60, were eluted with 0.1 M acetate buffer containing 15, 40, and 60% 1-propanol, respectively. As judged from their recoveries (*ca.* 30, 45, and 10%, based on the crude LTA) and fatty acid contents, BOS15, BOS40, and BOS60 were assumed to correspond to NA, LTA-1, and LTA-2 in the previous work, respectively. The IL-6 inducing activities of BOS40 and BOS60 also supported this assumption. BOS40 and BOS60 induced IL-6 to similar extent to those of LTA-1 and LTA-2, respectively, as tested with human peripheral whole blood cells (Fig. 1) in the same manner as described previously (*13*). Because of its higher yield, BOS40 was used for further fractionation in the present study.

Further fractionation of BOS40 was performed by ion-exchange membrane chromatography on QMA-Mem Sep 1010 as in the previous study (*13*) but with a stepwise elution method. Two fractions, QM-A and QM-I, were obtained from eluates with 0.01 M acetate buffer containing 35% 1-propanol and the same buffer containing both 35% 1-propanol and 1 M NaCl, respectively. IL-6 inducing activity was found in QM-A, but not in QM-I (Fig. 2), in good agreement with our previous work (*13*).

QM-I was eluted as a broad but single peak on a gel permeation HPLC. Its molecular weight was estimated to be in the range of $2\text{-}8\times10^3$ (calibrated by the use of standard heparins) or $7\text{-}14\times10^3$ (calibrated by the use of standard dextrans). QM-I is also hydrophobically and anionically homogeneous as judged from the elution profile in the corresponding chromatographies. QM-I can therefore be regarded as a homologous high-molecular glycoconjugate with the above-mentioned molecular weight range. Its chemical composition is summarized in Table I.

No liberated free phosphate was detected on direct alkaline phosphatase digestion of QM-I, the presence of phosphomonoester being excluded. The phosphorus in the molecule was thus concluded to exist in the phosphodiester form. The molar ratio of phosphorus to glycerol was approximately 1, suggesting the existence of the poly(glycerophosphate) structure described for LTA. To cleave phosphodiester linkages expected to be abundantly present in the molecule, QM-I was digested by hydrofluoric acid (HF) hydrolysis and the products were partitioned with a mixture of chloroform/methanol/water (2 : 1 : 3, v/v/v). On TLC analysis using solvent system A, one major and several minor products were detected in the organic phase of the HF-hydrolysate (Fig. 3). They were isolated by preparative TLC. Their FAB-MS and ^{1}H NMR spectra revealed that the major product has a Glc_2acyl_2Gro structure, and the minor ones are the lyso derivative, diacylglycerol, and fatty acids.

One-dimensional ^{1}H NMR data for the glycerol and glucose residues in Glc_2acyl_2Gro are summarized in Table

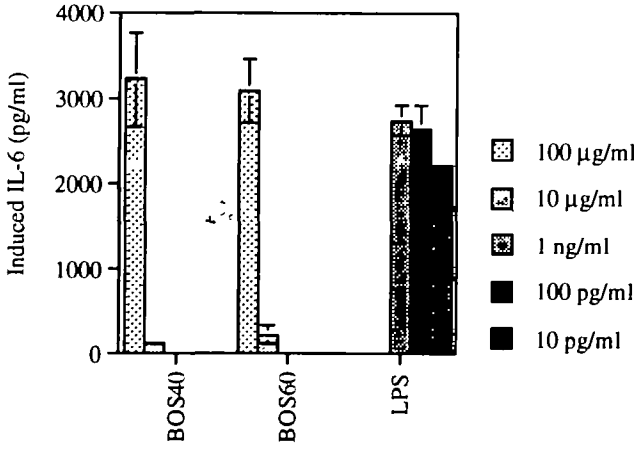

Fig. 1. **IL-6 inducing activity of BOS40 and BOS60.** Doses of samples were 100, 10 μg/ml for BOS40 and BOS60 and 1000, 100, 10 pg/ml for LPS. Data are means±SD of two experiments. The blood donor was JY.

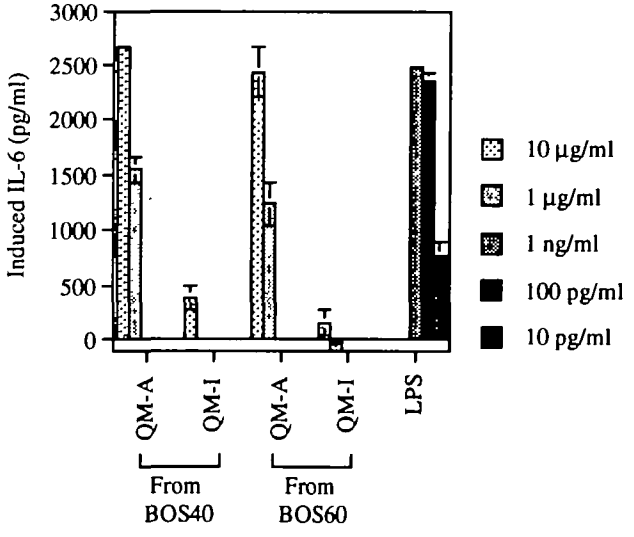

Fig. 2. **IL-6 inducing activity of QM-A and QM-I from BOS40 and BOS60.** Doses of samples were 10, 1 μg/ml for QM-A and QM-I and 1000, 100, 10 pg/ml for LPS. Data are means±SD of two experiments. The blood donor was MH.

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II. The coupling constants of the protons at the 1 to 4-positions of both glucose residues indicated pyranosyl ring structures (*17*). The chemical shifts (δ 4.98 and 4.95) and the coupling constants (3.5 and 3.8 Hz as $^3J_{1,2}$) of the anomeric protons confirmed the α-anomeric configurations (*18*). With regard to the glycerol residue, the downfield shifts of the H-1 and H-2 in comparison with the H-3 indicated that the fatty acids are located at these 1- and 2-positions. In the NOESY spectrum of QM-I shown in Fig. 4, interresidual NOEs between Glc^{nr} H-1 and Glc^{r} H-2, and Glc^{r} H-1 and Gro H-3 were observed (Glc^{nr} stands for the non-reducing side glucose and Glc^{r} for the reducing side glucose). On peracetylation, no downfield shift of Glc^{r} H-2 was observed in the ^{1}H NMR spectrum (data not shown). From these results, the glycolipid structure was determined as Glc^{nr}(α1-2)Glc^{r}(α1-3)acyl$_2$Gro. These NMR data were in good agreement with those of a synthetic counterpart (*9*) cited in Table II. The results also prove the stereochemistry of the glycerol residue to be the same as that present in the common glycoglycerolipid.

The fatty acid esters in the glycolipid were mainly composed of hexadecanoate, hexadecenoate, octadecanoate, and octadecenoate in the molar ratio of 0.63 : 0.23 : 0.10 : 1.00. The positive ion mode FAB-MS analysis of the glycolipid portion showed the molecular ion peaks depicted in Fig. 5. The heterogeneity of the molecular weight is explained by the substitution with different combinations of fatty acids at the 1- and 2-positions of the glycerol: *e.g.*, the peak at m/z 942 corresponds to a glycolipid with one hexadecanoic and one octadecenoic acid, whereas the peak

TABLE I. **Chemical composition of QM-I.** Molar ratios were calculated by assuming the presence of two moles of fatty acids.

	Wt%	(Molar ratio)
Phosphate	11.5	(12.0)
Glycerol	18.7	(14.0)
Glucose	42.4	(17.3)
Fatty acid	7.6	(2.0)
C16:0		(0.7)
C16:1		(0.1)
C18:0		(0.2)
C18:1		(0.9)
Alanine	2.3	(2.0)

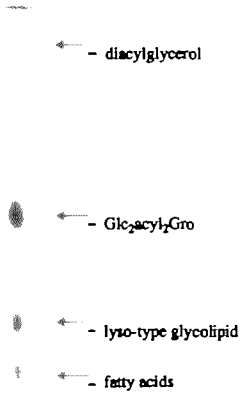

Fig. 3. **TLC analysis of the products extracted in the organic phase from the HF-hydrolysate of QM-I.** TLC was done on a silica gel plate (Merck Keiselgel 60 F_{254} Art. 5715) using solvent system A; chloroform/methanol/water (65 : 25 : 4, v/v/v). The products were visualized with an anisaldehyde-sulfuric acid reagent, and scanned by an image scanner (ScanJet IIcx, Hewlett Packard).

TABLE II. **^{1}H NMR data for the glycolipid Glc$_2$acyl$_2$Gro obtained by HF-hydrolysis of QM-I and its synthetic counterpart.** The spectra were measured in $CDCl_3$–CD_3OD (3 : 1) at 303 K. The assignments were established by ^{1}H and ^{13}C one-dimensional spectroscopy and the following two-dimensional methods: DQF-COSY, TOCSY, and HMQC. Glc^{r} and Glc^{nr} stand for the reducing and non-reducing side glucose residues, respectively. The data for synthetic glycolipid were taken from Ref. *9*.

Proton	Glc$_2$acyl$_2$Gro		Synthetic glycolipid	
	Chemical shift ($^3J_{H,H}$)		Chemical shift ($^3J_{H,H}$)	
	δ	(Hz)	δ	(Hz)
Glycerol				
Gro H-1	4.20	(6.6, 12.2)	4.20	(6.4, 12.1)
	4.43	(3.4, 12.2)	4.43	(3.0, 12.1)
Gro H-2	5.23		5.23	
Gro H-3	3.64	(5.1, 10.8)	3.64	(5.3, 10.6)
	3.83	(5.3, 10.9)	3.83	(5.8, 10.6)
Glucose				
Glc^{r} H-1	4.98	(3.5)	4.98	(3.6)
Glc^{r} H-2	3.59	(3.5, 9.8)	3.58	(3.6, 9.6)
Glc^{r} H-3	3.78	(8.9, 9.8)	3.78	
Glc^{r} H-4	3.45	(8.9, 9.7)	3.44	(9.2, 9.9)
Glc^{r} H-5	3.57		3.57	
Glc^{r} H-6	3.78		3.7-3.8	
Glc^{nr} H-1	4.95	(3.8)	4.95	(3.8)
Glc^{nr} H-2	3.45	(3.9, 9.6)	3.44	(3.8, 9.5)
Glc^{nr} H-3	3.70	(9.0, 9.6)	3.69	(9.2, 9.5)
Glc^{nr} H-4	3.33	(9.1, 9.8)	3.34	(9.2, 9.5)
Glc^{nr} H-5	3.88		3.88	
Glc^{nr} H-6	3.69	(6.1, 12.2)	3.70	(6.2, 12.4)
	3.86		3.86	

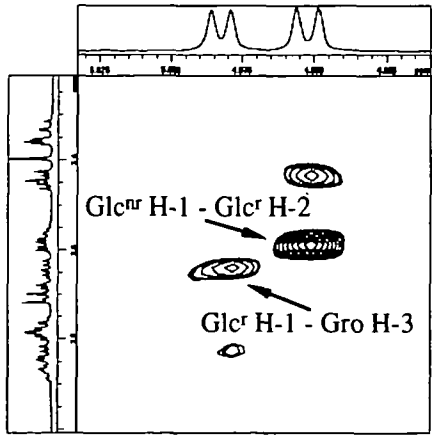

Fig. 4. **A part of the NOESY spectrum of Glc$_2$acyl$_2$Gro.** The spectrum was measured in chloroform/methanol (3 : 1) at 303 K. The mixing time was 650 ms.

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at m/z 968 contains two octadecenoic acids.

The products extracted in the aqueous phase from the HF-hydrolysate of QM-I were analyzed by ESI-MS. In the positive ion mode, two series of ion peaks at m/z 277.2, 439.3, 601.2, 763.4, 925.4 and m/z 326.2, 488.3, 650.4, 812.4 were observed (Fig. 6). The former series was assigned to sodium adducts of Glc_nGro ($n=1$–5) and the latter to $AlaGlc_nGro$ ($n=1$–4) in its protonated form. Since the negative ion mode ESI-MS indicated the presence of compounds which still retained phosphate groups, the mixture was further treated with an alkaline phosphatase to remove the remaining phosphates. In the ESI-MS analysis (positive ion mode) of the resulting dephosphorylated products, only the molecular ion peaks of the sodium adducts of Glc_nGro ($n=1$–5) were observed and the alanine substituents were no longer detected. Since the phosphatase treatment was undertaken under weakly alkaline conditions, the disappearance of the alanine residues suggests that they were linked to Glc_nGro through ester linkages. The dephosphorylated products were peracetylated and five compounds were isolated by preparative TLC using solvent system B (the pattern of analytical TLC is shown in Fig. 7). The isolated compounds were characterized on the basis of ^{1}H-NMR spectra as peracetylated Gro, $Glc^I(\alpha 1\text{-}2)Gro$, $Glc^{II}(\alpha 1\text{-}2)Glc^I(\alpha 1\text{-}2)Gro$, $Glc^{III}(\alpha 1\text{-}2)Glc^{II}(\alpha 1\text{-}2)Glc^I(\alpha 1\text{-}2)Gro$, and $Glc^{IV}(\alpha 1\text{-}2)Glc^{III}(\alpha 1\text{-}2)Glc^{II}(\alpha 1\text{-}2)Glc^I(\alpha 1\text{-}2)Gro$ (the glucosyl residues are numbered from the one linked to the glycerol to the non-reducing terminus). In spite of its detection in ESI-MS mentioned above, the presence of Glc_5Gro was not observed on TLC because of its low content. The ^{1}H NMR data of these products are summarized in Table III. The coupling constants of the anomeric and other ring protons indicated an α-glucopyranosyl configuration for all glucose residues. No downfield shifts of Gro H-2 were observed on peracetylation of Glc_nGro, indicating the $Glc(\alpha 1\text{-}2)Gro$ linkages. Downfield shifts were not observed for Glc^I H-2 and Glc^{II} H-2 of peracetylated Glc_3Gro either. This proves the $Glc(\alpha 1\text{-}2)Glc$ linkages in Glc_nGro. The positions of interresidual linkages were further confirmed by HMBC. Thus, the expected interresidual correlations in the peracetylated Glc_2Gro were observed between Glc^{II} C-1 and Glc^I H-2, Glc^{II} H-1 and Glc^I C-2, Glc^I C-1 and Gro H-2, and Glc^I H-1

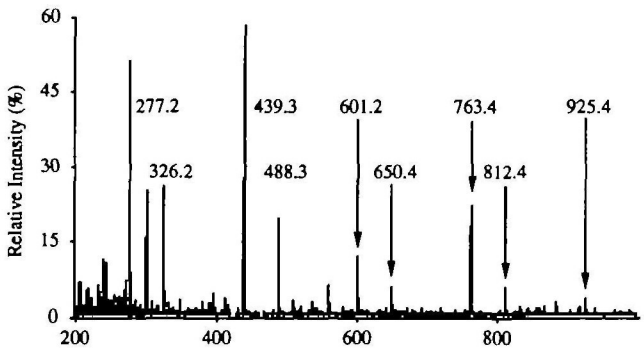

Fig. 6. **ESI-MS analysis of the products extracted in the aqueous phase from HF-hydrolysate of QM-I.** A 0.1 mg/ml solution (in 20% 2-propanol containing water) was continuously infused at 0.3 ml/h.

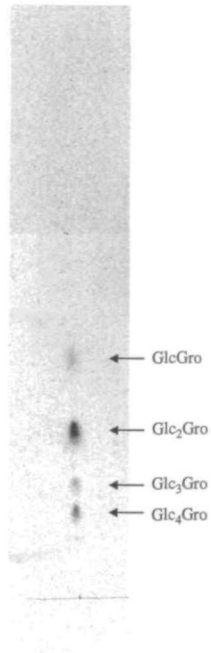

Fig. 7. **TLC analysis of the products extracted in the aqueous phase from the HF-hydrolysate of QM-I.** TLC was done on a silica gel plate (Merck Keiselgel 60 F_{254} Art. 5715) using solvent system B; chloroform/methanol/ethyl acetate (100 : 4 : 6, v/v/v). The products were visualized with an anisaldehyde-sulfuric acid reagent, and scanned by an image scanner (ScanJet IIcx, Hewlett Packard). Peracetylated Gro, not visualized by this method, was detected with iodine vapor (data not shown).

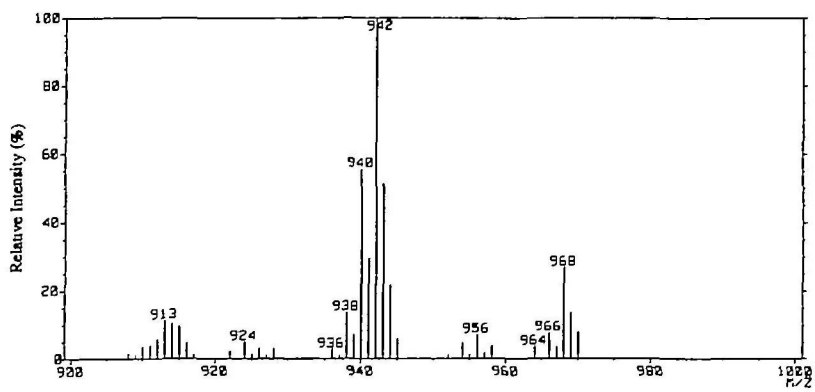

Fig. 5. **FAB-MS analysis of Glc_2acyl_2Gro.** *m*-Nitrobenzyl alcohol was used as a matrix.

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and Gro C-2.

To elucidate the mode of connection between the glycolipid and the Glc$_n$Gro moiety, the NMR spectra of deacylated QM-I were examined. The ^{13}C NMR spectrum is shown in Fig. 8. The signals at δ 67.38 and 67.52 with coupling constants of *ca.* 5 Hz are assigned to Gro C-1 and -3. Their downfield shifts and the coupling constants corresponding to a value expected for $^2J_{C,P}$ indicate phosphodiester linkages at the 1 and 3-positions of glycerol and, therefore, prove the presence of a 1,3-phosphodiester-linked poly-

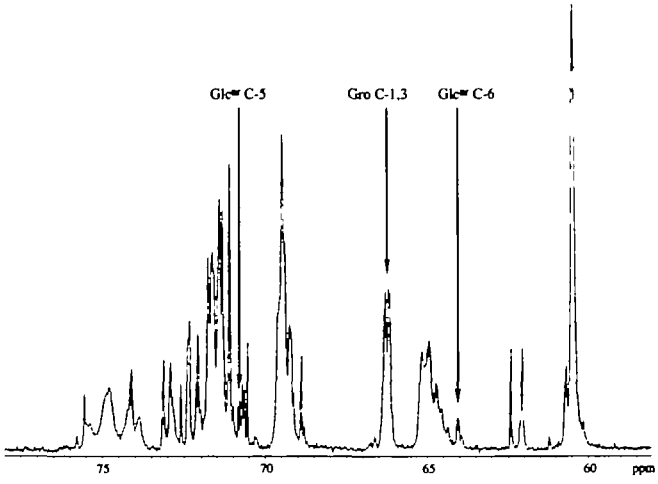

Fig. 8. **^{13}C NMR spectrum of deacylated QM-I.** The spectrum was measured in D_2O at 303 K. The assignments were established by DEPT (135°), HMQC, HMBC, DQF-COSY, TOCSY, and NOESY. Glc^{nr} means the non-reducing side glucose of the glycolipid region.

TABLE IV. **^{1}H and ^{13}C NMR data for the glycerol and glucose residues of the glycolipid region in deacylated QM-I.** The spectra were measured in D_2O at 303 K. The assignments were established by ^{1}H and ^{13}C one-dimensional spectroscopy and the following two-dimensional methods: DQF-COSY, TOCSY, NOESY, HMQC, and HMBC, as well as by reference to the data in Ref. *16*. Glc^r stands for the glucose residue of the reducing side, Glc^{nr} for that of the non-reducing side.

Position	Chemical shifts (δ)	
	^{1}H	^{13}C
Gro 1	3.65	64.62
Gro 2		71.52
Gro 3	3.81	70.00
Glc^r 1	5.11	97.15
Glc^r 2	3.63	76.74
Glc^r 3	3.76	72.55
Glc^r 4	3.42	70.69
Glc^r 5	3.54	72.55
Glc^r 6	3.67	61.88
	3.76	
Glc^{nr} 1	5.04	97.40
Glc^{nr} 2	3.56	72.33
Glc^{nr} 3	3.73	73.80
Glc^{nr} 4	3.50	70.69
Glc^{nr} 5	3.97	72.01
Glc^{nr} 6	4.06	65.25

TABLE III. **^{1}H NMR data for peracetylated Gro, GlcGro, Glc$_2$Gro, Glc$_3$Gro, Glc$_4$Gro.** The spectra were measured in $CDCl_3$ at 303 K. The assignments were established by ^{1}H and ^{13}C one-dimensional spectroscopy, DQF-COSY, and HMQC. Glucosyl residues are numbered from that linked to the glycerol to the non-reducing terminus.

Proton	Chemical shifts δ (Coupling constants) (Hz)				
	Gro	GlcGro	Glc$_2$Gro	Glc$_3$Gro	Glc$_4$Gro
Gro H-1	4.16 (5.7, 11.9)	4.09	4.18	4.18	4.09
	4.29 (4.1, 11.9)	4.27	4.28	4.38	4.43
Gro H-2	5.24	4.06	4.09	4.26	4.18
Gro H-3	4.16 (5.7, 11 9)	4.09	4.21	4.17	4.24
	4.29 (4.1, 11 9)	4.27	4.27	4.33	4.30
Glc^I H-1		5.31 (3.9)	5.21 (3.7)	5.33 (3.9)	5.34 (3.9)
Glc^I H-2		4.84 (3.8, 10.3)	3.75 (3.6, 9.9)	3.73 (3.9, 10.1)	3.93 (3.7,9.9)
Glc^I H-3		5.47 (9.6, 10.1)	5.45 (9.4, 9.8)	5.45 (9.6, 9.6)	5.49 (9.4,9.6)
Glc^I H-4		5.06 (9.8, 9.9)	4.98 (9.2, 10.3)	5.00 (9.6, 9.9)	5.04 (9.1, 9.4)
Glc^I H-5		4.20	4.21	4.26	4.27
Glc^I H-6		4.17	4.05	4.06	4.11
		4.23 (4.6, 12.4)	4.29		
Glc^{II} H-1			5.16 (3.5)	5.04 (3.2)	5.05 (3.0)
Glc^{II} H-2			4.87 (3.5,10.4)	3.76 (3.2, 10.3)	3.70 (3.2, 10.3)
Glc^{II} H-3			5.37 (9.5, 10.2)	5.40 (9.6, 9.9)	5.33
Glc^{II} H-4			5.07 (9.5, 10.2)	4.99 (9.6, 10.1)	5.00 (9.6, 9.9)
Glc^{II} H-5			4.07	4.16	4.04
Glc^{II} H-6			4.16	4.07	
				4.18	
Glc^{III} H-1				5.10 (3.7)	5.20 (3.4)
Glc^{III} H-2				4.94 (3.7, 10.3)	3.76 (3.4, 9.9)
Glc^{III} H-3				5.32 (9.4, 10.3)	5.35
Glc^{III} H-4				5.08 (9.4, 10.1)	5.01 (9.6, 10.0)
Glc^{III} H-5				4.11	4.08
Glc^{III} H-6				4.18	4.17
Glc^{IV} H-1					5.12 (3.9)
Glc^{IV} H-2					4.92 (3.6, 10.2)
Glc^{IV} H-3					5.31
Glc^{IV} H-4					5.10 (9.6, 10.1)
Glc^{IV} H-5					4.00
Glc^{IV} H-6					4.19

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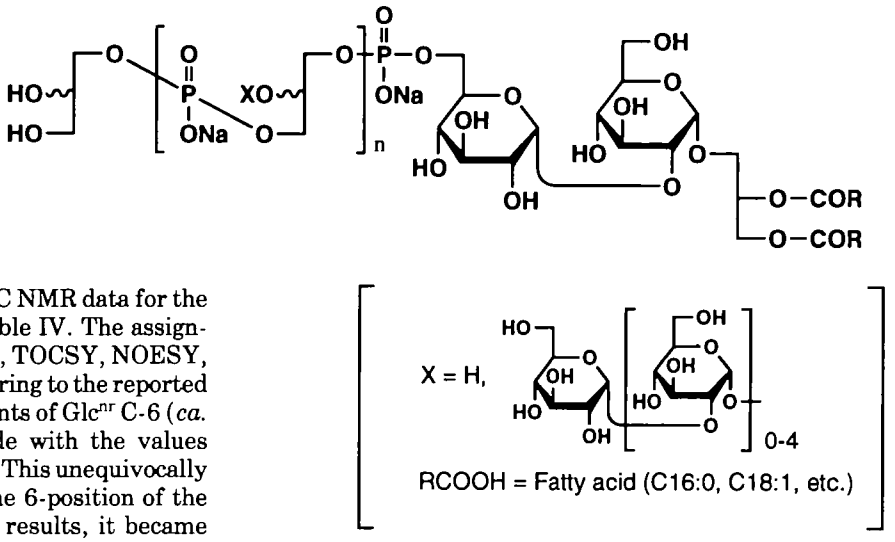

Fig. 9. **The structure of QM-I from BOS40.** A part of the oligoglucosyl substituents on glycerols may be further substituted with alanine.

(glycerophosphate) chain. The ^{1}H and ^{13}C NMR data for the glycolipid region are summarized in Table IV. The assignments were established by DEPT (135°), TOCSY, NOESY, HMQC, and HMBC spectra and by referring to the reported data (*16*). The observed coupling constants of Glc^{nr} C-6 (*ca.* 5 Hz) and Glc^{nr} C-5 (*ca.* 7 Hz) coincide with the values expected for $^{2}J_{C,P}$ and $^{3}J_{C,P}$, respectively. This unequivocally proves the phosphodiester linkage at the 6-position of the non-reducing side glucose. From these results, it became evident that the poly(glycerophosphate) chain is linked to the 6-position of the non-reducing side glucose in the glycolipid moiety.

Taking all the data into account, the chemical structure of QM-I, the major but biologically inactive component in BOS40 from *E. hirae* ATCC 9790, was deduced to be as shown in Fig. 9, except for the location of the alanyl substitution and the stereochemistry of the glycerols, which are still under investigation. The structure thus concluded coincides with the one proposed by Fischer's group for LTA-1 isolated from the same bacteria. These apparently identical conclusions, however, have completely different implications. Fischer's group had first studied the structure of major components of LTA regardless of their biological activities. In their later publications (*7, 19*), the immunostimulating activity of the whole LTA is described again without separation. Such an approach might lead to the incorrect understanding that the chemical entities whose structures had been elucidated by them were responsible for the activity. By contrast, our present work has unequivocally demonstrated the chemical structure of the major component, which was proved to be biologically inactive in our test system with human cells, of LTA from *E. hirae* ATCC 9790. This means that the immunostimulatory minor components of LTA isolated by us must be structurally different from the major component shown in Fig. 9. In other words, the presence of unsaturated fatty acids, long chain poly(glycerophosphate), or glucose or alanine substituents on the glycerol as discussed in the introduction, is irrelevant to the lack of biological activity of the recently synthesized fundamental structures of LTA (*9-11*).

The major component in BOS60 which is more lipophilic than BOS40 was also biologically inactive (Fig. 2). The chemical structure of this inactive component has not been studied by ourselves, but we tentatively assume it to be identical to LTA-2 which has, as proposed by Fischer, an additional phosphatidyl substituent at the 6-position of the reducing side glucose of the glycolipid part of LTA-1 (*1*). Chemical characterization of the active components in QM-A from both BOS40 and BOS60 is in progress in our laboratory.

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