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Structural Characterization of Monophosphoryl Lipid A Homologs Obtained from *Salmonella minnesota* Re595 Lipopolysaccharide*

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Sixteen monophosphoryl Lipid A (MLA) homologs obtained from the lipopolysaccharides of *Salmonella minnesota* Re595 were separated by preparative thin layer chromatography into eight fractions. The components of these fractions were analyzed directly (or as structural analogs) and characterized by mass spectrometry. Molecular weights were determined by negative and positive ion fast atom bombardment mass spectrometry and component structures were assigned following a study of fragmentation and metastable ion kinetic energy spectrometry. One fraction (TLC-8) contained a single heptaacyl MLA of M_r = 1,954, a structure previously elucidated (Qureshi, N., Mascagni, P., Ribi, E., and Takayama, K. (1985) *J. Biol. Chem.* 260, 5271–5278). The remaining seven fractions contained 15 additional MLAs with decreasing acylation. Two of these components have been previously reported in *S. minnesota* and *Salmonella typhimurium*. Three of the eight TLC fractions (TLC-8, -7, -6) were found to be biologically active toward human platelets inducing their aggregation and secretion of serotonin. All tested fractions induced varying degrees of phosphorylation of a platelet protein of M_r = 47,000 (P47) reflecting protein kinase C activation (Grabarek, J., Her, G. R., Reinhold, V. N., and Hawiger, J. J. (1990) *J. Biol. Chem.* 265, 8117–8121).

Endotoxic Lipid A is responsible for many of the pathophysiological effects of Gram-negative bacteria and it has many cellular targets. These include blood platelets, granulocytes, monocytes, lymphocytes, erythrocytes, and endothelial cells (1–3). Much attention has been focused on lethal toxicity of endotoxic lipopolysaccharide, its pyrogenicity, immunomodulating activities, and those factors that induce various mediators (4). However, such complications of Gram-negative bacteremia as thrombocytopenia and disseminated intravascular coagulation in septic shock involve human platelets (5, 6). It has been recently demonstrated that platelets are directly simulated by endotoxic Lipid A via protein kinase C pathway (7).

Lipid A serves as the lipophilic membrane anchor for the outer polysaccharide coat of Gram-negative bacteria. Structural studies of the Lipid A moiety obtained from *Salmonella typhimurium* G30/C21 has indicated the lipophilic anchor to be a homologous mixture differing in the number of attached fatty acid residues (8–11) and this heterogeneity appears to be the case with other species (12–14). In *Salmonella minnesota* Re595, the heptaacyl monophosphoryl Lipid A (MLA)[1] structure, has been characterized as a phosphorylated 2-amino-2-deoxy-D-glucose disaccharide linked $\beta(1\rightarrow6)$ (15) with a (*R*)-3-hydroxytetradecanoyl group on the 2,3-position (GlcN I), and 2′,3′-position (GlcN II). Additionally, the hydroxyl group on three of these residues (at positions 2,2′3′) are esterified with palmityl, lauryl, and myristyl groups, respectively (16). Two other analogs were also isolated from this rough mutant and they were shown by FAB-MS to be identical with the hexa- and pentaacyl analogs obtained from *S. typhimurium* (12).

The structures of the hepta-, hexa-, and pentaacyl MLA obtained from *S. minnesota* Re595 lipopolysaccharide were determined by FAB-MS and with the combined use of nuclear magnetic resonance spectroscopy; a detailed structure of the heptaacyl MLA has been presented (16). The molecular weights for these three components were determined by positive ion extraction of the phosphate-methylated, high performance liquid chromatography-purified MLAs. Structural detail was assigned from a glycosidic cleavage fragment which, in combination with *N*- and *O*-acyl analysis, indicated the structures to be identical with those detected in *S. typhimurium* (10). Earlier studies with this glucosamine disaccharide had indicated a $\beta(1\rightarrow6)$ linkage and the presence of a 4′-phosphate group (10–12). This excellent work (8–16) has provided the basis for a more detailed examination of all the Lipid A homologs present in these extracts.

We have been most interested in the stimulation exhibited by human platelets (17) upon incubation with Lipid A, and this study has focused on a structural characterization of Lipid A homologs responsible for interaction with platelet membranes. Herein, we characterize the components in each TLC fraction by direct analysis using FAB-MS and MIKES and augment these studies with chemical modification and reanalysis of the derivatives. In addition to the single heptaacyl MLA, three hexa-, four penta-, four tetra-, three tri-, and one diacyl homolog have been characterized in these *S. minnesota* Re595 Lipid A extracts. In the following paper (17)

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[1] The abbreviations used are: MLA, monophosphoryl Lipid A; FAB-MS, fast atom bombardment mass spectrometry; MIKES, metastable ion kinetic energy spectrometry; OHC_{14}, hydroxymyristoyl group; C_{12}, lauroyl group; C_{14}, myristoyl group; $C_{14}OC_{14}$, myristoxymyristoyl group.

TLC-purified MLA fractions are studied for their biological activity in terms of the aggregation of platelets, the release of [^{14}C]serotonin, and phosphorylation of a platelet substrate for protein kinase.

EXPERIMENTAL PROCEDURES

Mass Spectrometry—Fast atom bombardment was performed on a VG ZAB-SE instrument (VG Analytical, Manchester, United Kingdom) operated at 8 and 10 kV in the negative and positive modes, respectively. The FAB gun was operated at 8 kV using xenon as the ionizing gas. Current controlled scans were acquired at a rate of 30 s/decade, and 3–5 scans were summed as continuous data before converting to the bar plots down. The resolution was typically about 1:2500; CsI clusters were used for calibration. The selection and variation of the FAB matrix and ion extraction modes were important for obtaining complete spectral detail of each sample. For molecular weight information, abundant parent ions were obtained using triethanolamine and thioglycerol in the negative and positive ion modes, respectively. This matrix was frequently used to assess the number of components in each TLC fraction, since fragmentation seems to be diminished under these conditions. As discussed below, this strategy may not always be successful for the less lipophilic materials. For greater structural detail, (*i.e.* fragmentation), and lower backgrounds thioglycerol was replaced with *m*-nitrobenzylalcohol (MNBA) (18). For these samples the inclusion of MNBA in the matrix has proven to be an exceptionally useful aprotic dispersing agent that provides a combination of abundant ions with structural and molecular weight information. Moreover, operation in the negative ion mode usually provides spectra free of sodium or potassium ion adducts which causes signal dispersal. The sample concentration in the matrix was ~0.5–1.0 nmol/μl.

Mass analyzed ion kinetic energy spectra were acquired by focusing the precursor ions into the collision cell (second field free region) and scanning the product ions with the electric sector at a rate of 30 s/scan. Metastable ions were detected in the absence of a collision gas. As a result, mass assignments were more accurate, presumably because no kinetic energy loss was suffered.

Fractionation of Lipid A Mixture—Lipid A of *S. minnesota* Re595 (Calbiochem) was dissolved in chloroform and fractionated by TLC (19). Silica gel plates (1000 μm; Whatman PLK 5), prerun in acetone, were used for separating Lipid A preparations. The solvent system consisted of chloroform/methanol/water (10:5:1, v/v). The solvent system used in separating *Escherichia coli* diphosphoryl Lipid A consisted of chloroform/methanol/water/glacial acetic acid (50:25:4:0.15, v/v). The dried chromatograms were observed under UV light (375 nm) and lipid spots, appearing as green fluorescent areas, were marked. The spots were scraped into vials and eluted with 10 ml of the solvent used for development. They were homogenized on a vortex mixer and then stirred for 30 min at 40 °C. After settling for 5 min, the supernatant was decanted and the residual gel was washed twice with 2 ml of chloroform/methanol mixtures of 1:1, 2:1, and 4:1 (v/v). The extracts were filtered (LID/X Filter Syringes ORG.45, Xydex Corporation) and evaporated to dryness under a stream of N_2 or under reduced pressure at 40–50 °C (Speed-Vac concentrator, Savant). The lipid residues were dissolved in chloroform and stored at −40 °C.

De-O-acylation—De-*O*-acetylation was carried out by suspending the samples (10–100 μg) in 0.5 ml of ammonia-saturated methanol for 30 min at 65 °C (20) or heating with triethanolamine in H_2O for 10 min at 100 °C (10). This latter method induced a greater degree of acyl elimination.

RESULTS

Fractionation of Lipid A—It has been shown previously, by chromatographic analysis of Lipid A preparations, that Lipid A consists of several species (19). Preparative thin-layer chromatography of chloroform-soluble Lipid A from *S. minnesota* resulted in several fractions, representing different molecular forms of Lipid A. The TLC mobilities ranged in R_F values from 0.35 (TLC-1) to 0.81 (TLC-8) as compared to R_F 0.84 for phosphatidylethanolamine, run as a control lipid. As seen from the results below, the Lipid A species were primarily monophosphoryl with the phosphate group located at the C4′ position and sample heterogeneity could be attributed to variations in fatty acid substitution.

To evaluate the complexity of the initial Lipid A extract from *S. minnesota*, a portion of the unfractionated MLA was analyzed directly by FAB-MS using negative and positive ion extraction. The negative ion spectrum, using a thioglycerol:triethanolamine matrix, provided an abundant series of deprotonated ions, $(M - H)^-$, which allowed an evaluation of component molecular weights (Fig. 1). The ions at *m/z* 1715, 1505, 1323, 1279, and 1097 (all *m/z* values are reported as nominal masses) correspond in mass to the hexaacyl and lower acylated MLAs detected in *S. typhimurium* (10). The matrix used during FAB-MS analysis is known to greatly influence the resulting spectra (21). As an example, when the same sample (Fig. 1) was analyzed using the matrix MNBA:triethyanolamine (1:1), the spectrum in Fig. 2 was obtained. Under these conditions, the ion abundance maxima for this series of homologs shift to higher mass, provide an improved signal-to-noise ratio, and a previously undetected component at higher mass was observed, *m/z* 1953.

A comparison of the two spectra indicates a fair qualitative relationship, although the quantitative aspects are quite different. Within each spectra an important interrelationship exists indicating differences by one or more acyl groups. As an example, the ion *m/z* 1715 can be related to the heptaacyl $(M - H)^-$ by a palmityl group (Δ 238); the ion *m/z* 1743 by a myristyl group (Δ 210 Da); and the ion *m/z* 1727 by a hydroxymyristyl group (Δ 226). Strong support for this structural relationship was provided when the total mixture was de-*O*-acylated and re-analyzed by FAB-MS. One major ion was detected, *m/z* 871, indicating complete de-*O*-acylation to a single 2,2′-(*N*-hydroxymyristoyl) 2,2′-amino disaccharide (Scheme 1). When this product was analyzed for metastable ions, none were detected. Since the acyl groups in the dimethylphosphoryl heptaacyl MLA have been identified and their positions located (16), it is reasonable to assume that

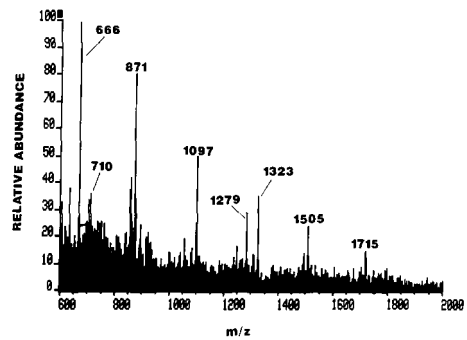


FIG. 1. **Direct FAB-MS analysis of unfractionated MLAs from *S. minnesota* Re595 using triethanolamine:thioglycerol (1:1) as a FAB matrix.**

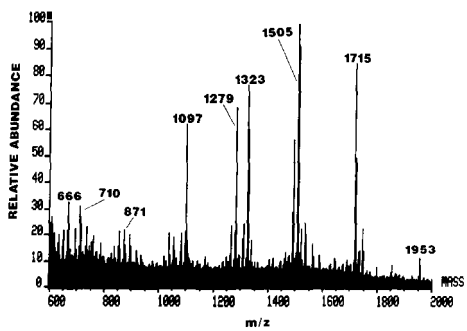
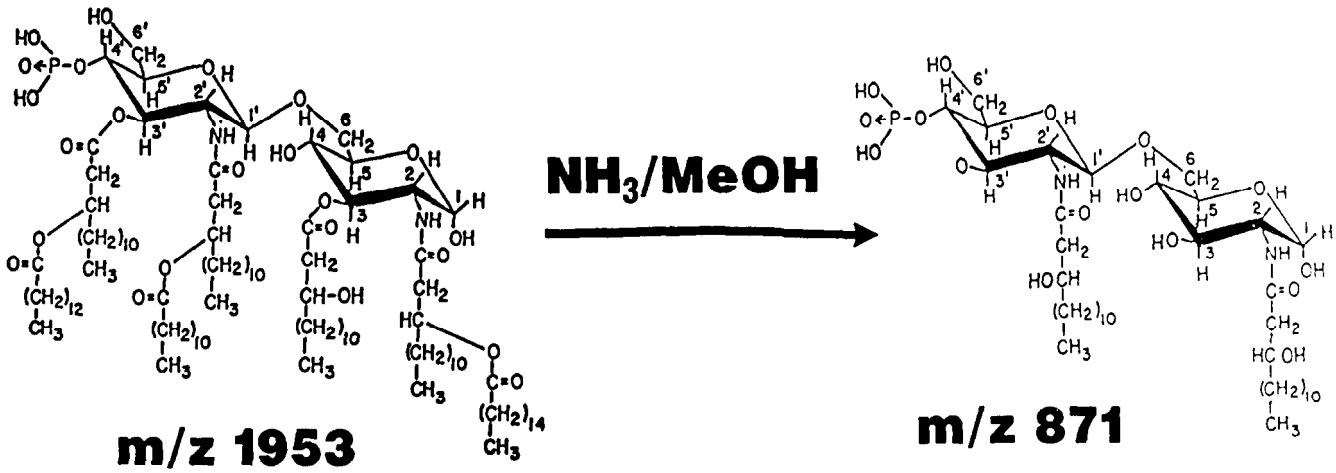


FIG. 2. **Direct FAB-MS analysis of unfractionated MLAs from *S. minnesota* Re595 using triethanolamine:metanitrobenzyl alcohol (1:1) as a FAB matrix.**



SCHEME 1. **Ammonia de-*O*-acylation.**

TABLE I

Structural summary of characterized components obtained by TLC fractionation of S. minnesota Re595 Lipid A

Molecular weight-related parent, $(M - H)^-$, with *O*-acyl (C-3 and C-3′) and *N*-acyl (C-2 and C-2′) structure at the four known positions of acylation. *X*°, terminal monosaccharide residue; X_e, elimination fragment; M, myristoyl; HM, hydroxymyristoyl; L, lauroyl; P, palmitoyl.

Ion structures					
$(M - H)^-$	C3′	C2′	C3	C2	TLC fraction
1953	M·HM	L·HM	HM	P·HM	8
1743	HM	L·HM	HM	P·HM	7
1727	M·HM	L·HM		P·HM	7
1715	M·HM	L·HM	HM	HM	7
1561	HM	HM	HM	P·HM	6
1533	M·HM	HM	HM	HM	6
1505	HM	L·HM	HM	HM	6
1489	M·HM	L·HM		HM	6
1323	HM	HM	HM	HM	5
1307	M·HM	HM		HM	5
1279		L·HM	HM	HM	5
1279	HM	L·HM		HM	3
1102°	M·HM	L·HM			Fragment
1097		HM	HM	HM	4
1097	HM	HM		HM	1
1079_e		HM	HM	HM	Fragment
1053		L·HM		HM	2
1035_e		L·HM		HM	Fragment
920°	M·HM	HM			Fragment
892°	HM	L·HM			Fragment
871°		HM		HM	4
710°	HM	HM			Fragment
694°	M·HM				Fragment

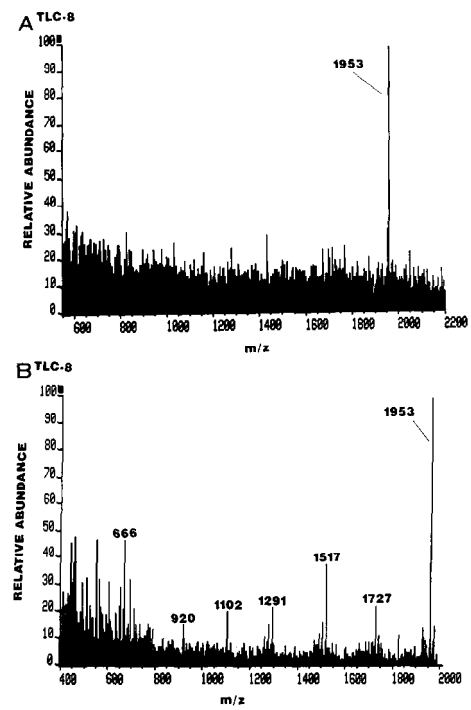
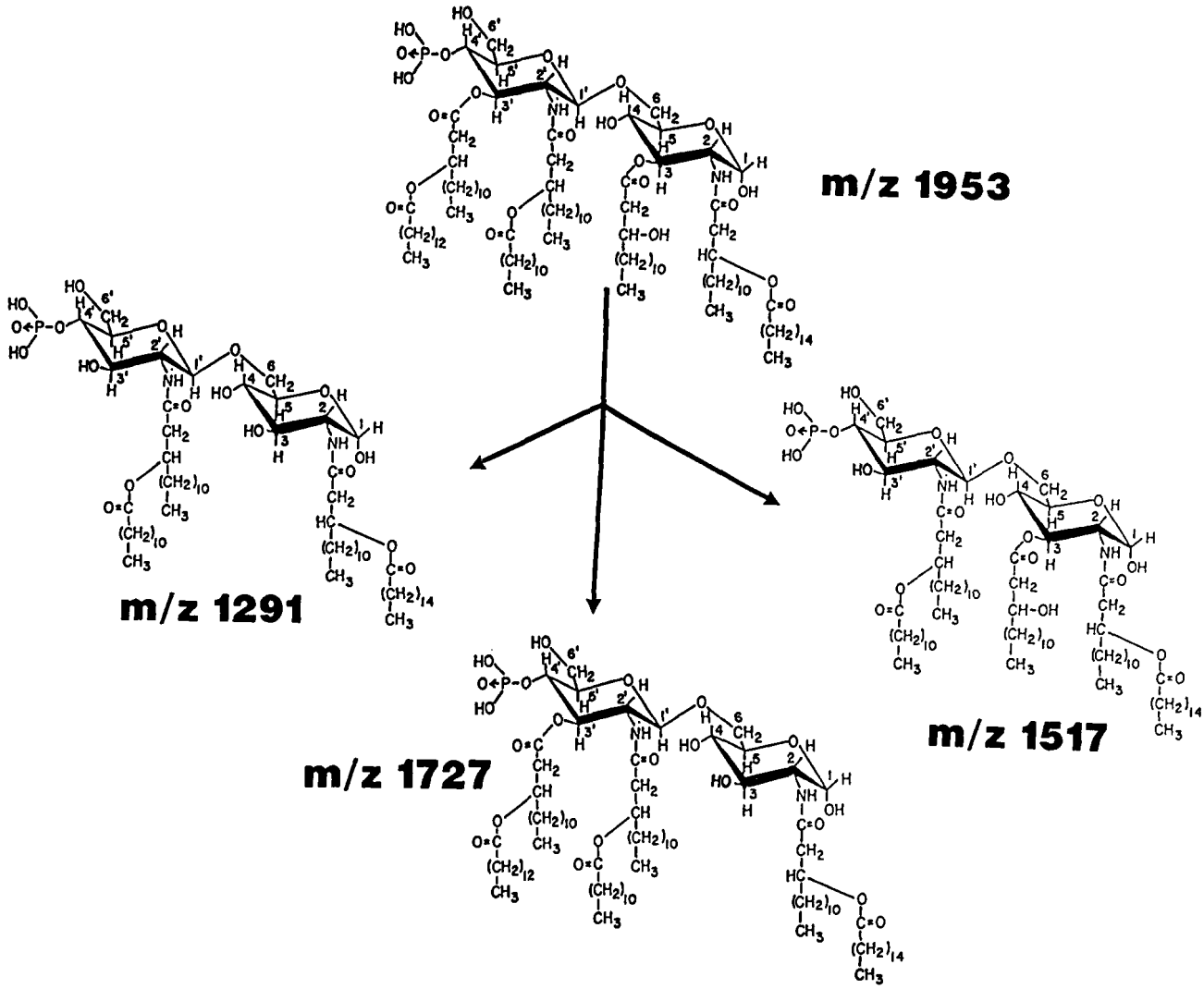


FIG. 3. *A*, fraction TLC-8 eluted with chloroform/methanol/water (10:5:1) and direct analysis by FAB-MS using triethanolamine:thioglycerol (1:1) as a FAB matrix. *B*, fraction, TLC-8, eluted with chloroform/methanol/water (10:5:1) and direct analysis by FAB-MS using triethanolamine:metanitrobenzyl alcohol (1:1) as a FAB matrix.

the isobaric components in these fractions are homologs with defined structure (Table I).

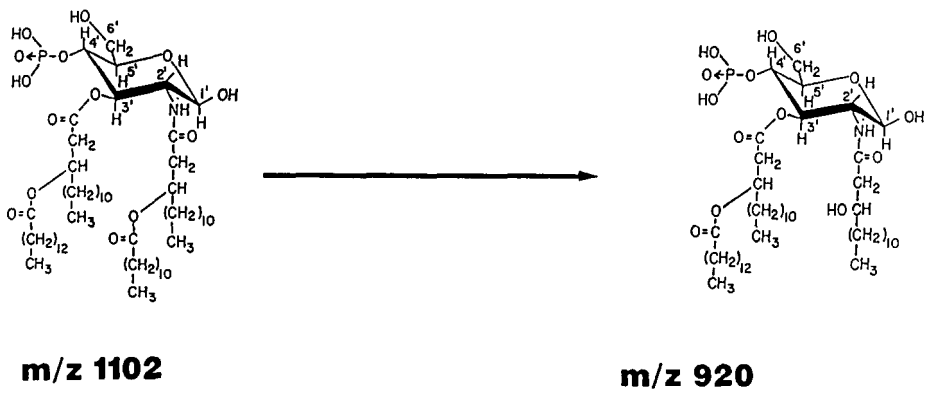
There are several low molecular weight ions in Fig. 1 that can not be related to the heptaacyl MLA by acyl group addition. However, they do correspond in mass to the terminal glycoside (GN-II) of the heptaacyl MLA and are themselves interrelated by a difference in acyl moieties. It could also be that these are not $(M - H)^-$ ions but fragments of higher molecular weight constituents; however, for the higher molecular weight MLAs, we have not observed this degree of fragmentation using the thioglycerol:triethanolamine matrix. Their occurrence here most likely reflects true components in the mixture and their structures are also listed in Table I.

All TLC fractions were analyzed directly by FAB using negative and positive ion extraction in two different matrices: thioglycerol (to maximize parent ion abundance) and MNBA:triethanolamine (to enhance fragmentation). The most lipophilic TLC spot (TLC-8) provided the negative FAB mass spectrum shown in Fig. 3*A*, indicating a single component with a molecular mass of 1954 Da. Corroboration of this molecular weight and important structural detail was provided when the sample was reanalyzed using the MNBA containing matrix (Fig. 3*B*). Under these conditions the single component provided three major fragments, *m/z* 1727, 1517, and 1291, which could be accounted for as acyl losses from the parent ion: a hydroxymyristoyl loss (1953 − 226 = 1727), a loss equal in composition to a myristoxymyristoyl group (1953 − 436 = 1517), and a combination of the above losses (1953 − (226 + 436) = 1291), respectively (Scheme 2). Since these fragments do not seem to involve the acyloxyhydroxymyristoyl residues that contain a palmitoyl or lauroyl group, this



SCHEME 2. **Heptaacyl MLA fragmentation (FAB-MS).**

SCHEME 3. **GlcN II fragmentation (FAB-MS).**



suggests the amide-linked groups are more stable. Two lower mass fragments, *m/z* 1102 and 920, are of considerable interest and provide structural insight into the terminal residue (GlcN II). The fragment *m/z* 1102 can be considered a cleavage on the reducing side of the glycosidic oxygen and losses from this terminal residue of a lauroyl group (Scheme 3). A third ion, *m/z* 666, detected in most TLC fractions, was initially thought to represent further loss of a myristoxymyristoyl group, but the ion was detected in blank TLC scrapings and cannot be considered an exclusive GlcN II fragment.

Following ionization, most molecules fragment immediately within the ion source, but some ions continue to dissipate their excess kinetic energy by slower fragmentation processes outside the ion source to produce what are called metastable ions. In multisector instruments, using a technique called metastable ion kinetic energy spectrometry (MIKES), it is possible to study every ion for metastable character. Focusing of individual precursor (parent) ions and detection of metastable product (daughter) ions provides additional insight to structure, and mass assignment identifies the groups involved. As an example, the heptaacyl MLA ion (*m/z* 1953) provided a metastable ion pair at *m/z* 1726 and 1709, which would

indicate a combined hydroxymyristoyl loss (as a ketene, Δ 226) and an elimination (as an acid, Δ 244) of the only possible residue with that mass at C3 (Fig. 4, Scheme 4). Consistent with this observation, metastable ions were not detected for hydroxymyristoyl loss from this molecule following de-*O*-acylation (see above, *m/z* 871), indicating that only the *O*-acyl hydroxymyristyl and not the C2 or C2′-*N*-acyl or C3′ acyloxyacyl groups were involved.

As summarized in Schemes 1–4, this combination of chemical de-*O*-acylation, FAB-induced rupture of acyl groups, glycosidic cleavage (providing terminal, and by difference proximal, molecular weights), and metastable character of the hydroxylmyristoyl group strongly suggests this material to be the hepataacyl MLA described earlier (16).

Analysis of the second biologically active spot, TLC-7, indicated three hexaacyl homologs: one major and two minor components, *m/z* 1715, 1727 (10%), and 1743 (20%), respectively. From the known structure of the heptaacyl MLA (1954 Da), and the mass difference of these ions (Δ 238, Δ 226, Δ 210), a unique structure may be represented for each (Table I). To study these ions in greater detail, the sample was analyzed under conditions that induce fragmentation (Fig. 5). The product ions, *m/z* 1489, 1279, 1053, and 1035, can be related to the most abundant component, *m/z* 1715, by respective losses of a hydroxymyristoyl group (Δ 226), a myristoxymyristoyl group (Δ 436), a combined myristoylmyristoyl and hydroxymyristoyl group loss (Δ 436 + 226 = 662), and a combined myristoylmyristoyl and hydroxymyristoyl group elimination, (436 + 244 = 680). Focusing and MIKES analysis of the *m/z* 1715 (Fig. 6*A*) and *m/z* 1743 (Fig. 6*B*) parent ions gave rise to a pair of metastable fragments for each parent ion, indicating acid elimination (Δ 244) and ketene cleavage (Δ 226) of a hydroxymyristoyl group. From the known parent ion structure, *m/z* 1715 (Table I) and the mass interval (Δ 244/226) to the metastable ion, it is concluded that only the C3 group is involved. This is in contrast to the *m/z* 1743 ion, where two such groups could contribute to the metastable ion (C3 and C3′) (Table I). A comparison of the ratios of acid elimination to ketene cleavage in the metastable ion product (Fig. 6*B*) indicates enhanced elimination in preference to cleavage. This may reflect the greater lability of the C3′ group to elimination induced by the C4′ phosphate group (Scheme 5).

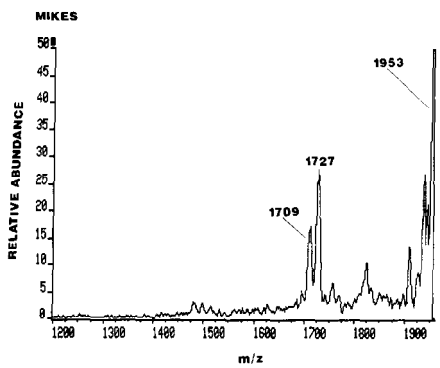
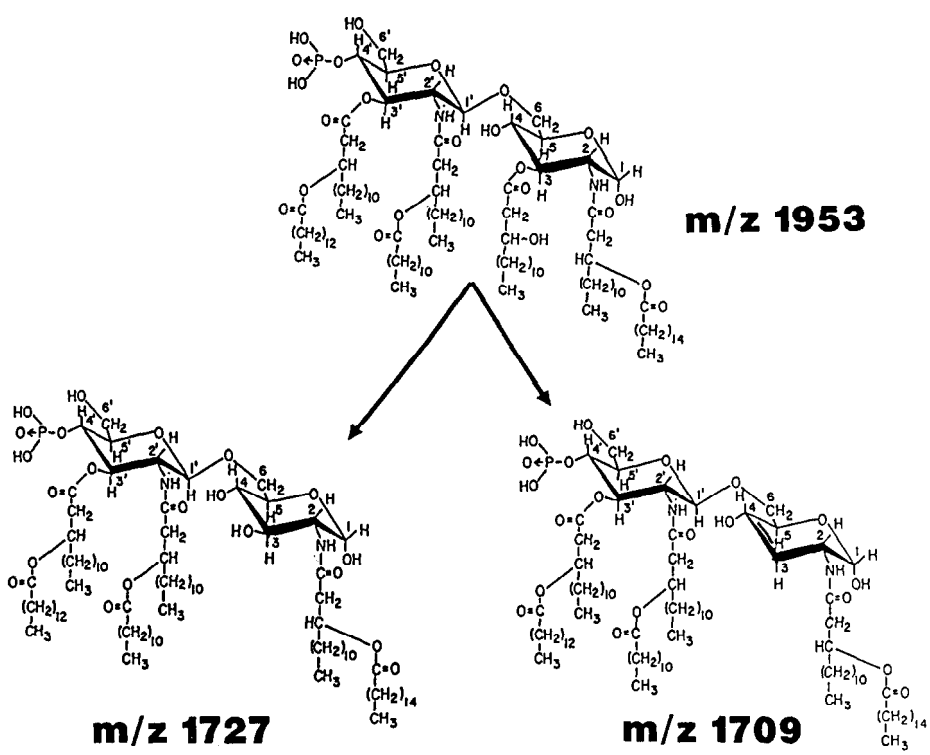


FIG. 4. **Metastable ion detected by focusing *m/z* 1953 (MIKES) indicating hydroxymyristic acid elimination (*m/z* 1709) and ketene loss (*m/z* 1727) (Δ 244 and 226, respectively).**

Mass spectral analysis of the third biologically active fraction, TLC-6, indicated four pentaacyl homologs. The most abundant pentaacyl component, *m/z* 1505, can be considered comparable in structure with the heptaacyl MLA but is missing the palmitoyl and myristoyl groups. The remaining three components, *m/z* 1489, 1533, and 1561, differ by a palmitoyl and hydroxylmyristoyl group (1953 − (238 + 226) = 1489), a palmitoyl and lauroyl group (1953 − (238 + 182) = 1533), and a lauroyl and myristoyl group (1953 − (182 + 210) = 1561) (Table I). Mass spectral analysis under fragmenting conditions (Fig. 7) provided several ions indicating acyl group and position for the most abundant component. The *m/z* 1261 fragment suggests hydroxylmyristoyl elimination (Δ 244) from the *m/z* 1505 parent and the C3′ location of this group is supported by the detection of an abundant metastable ion for acid elimination from the focused parent. The direct loss



SCHEME 4. **Heptaacyl MLA metastable ion products (MIKES).**

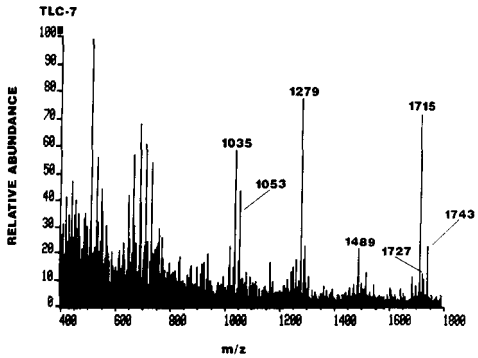


FIG. 5. **Fraction TLC-7 eluted with chloroform/methanol/water (10:5:1) and direct analysis by FAB-MS using triethanolamine:metanitrobenzyl alcohol (1:1) as a FAB matrix.**

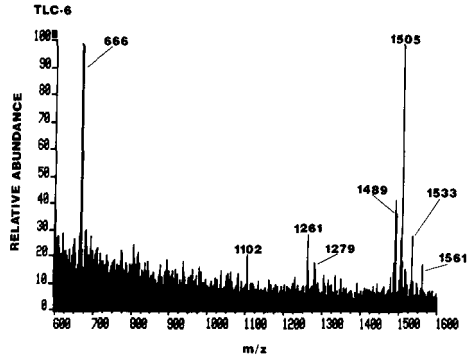


FIG. 7. **Fraction TLC-6 eluted with chloroform/methanol/water (10:5:1) and direct analysis by FAB-MS using triethanolamine:metanitrobenzyl alcohol (1:1) as a FAB matrix.**

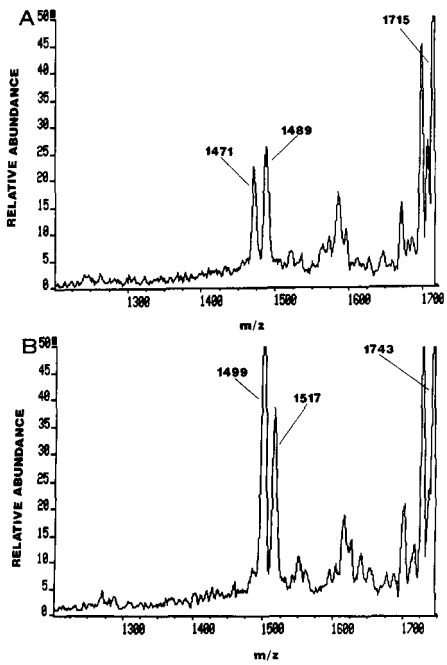
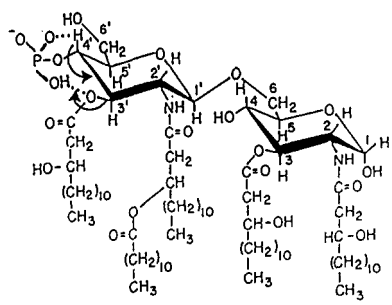


FIG. 6. *A*, metastable ion detected by focusing *m/z* 1715 (MIKES), indicating hydroxymyristic acid elimination (*m/z* 1471) and ketene loss (*m/z* 1489) (Δ 244 and 226, respectively). *B*, metastable ion detected by focusing *m/z* 1743 (MIKES), indicating hydroxymyristic acid elimination (*m/z* 1499) and ketene loss (*m/z* 1517) (Δ 244 and 226, respectively).



SCHEME 5

of a hydroxymyristyl group (Δ 226) may account for the fragment *m/z* 1279, although this may also be a product of a myristyl loss (Δ 210) from the *m/z* 1489 ion (Table I). The fragment at *m/z* 1102 indicates glycosidic cleavage with both acyl residues at C2′ and C3′ intact indicating the source of this fragment to be the *m/z* 1489 ion. A second glycosidic cleavage fragment in low abundance can be observed at *m/z*

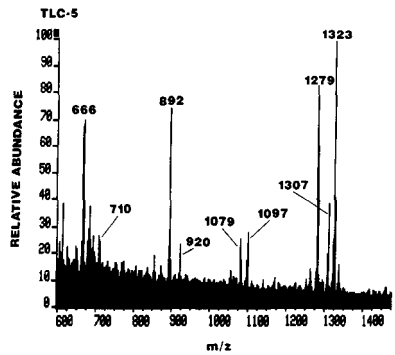


FIG. 8. **Fraction TLC-5 eluted with chloroform/methanol/water (10:5:1) and direct analysis by FAB-MS using triethanolamine:metanitrobenzyl alcohol (1:1) as a FAB matrix.**

892 indicating its origin to be the *m/z* 1505 ion.

The extract obtained from TLC-5 indicted three tetraacyl MLAs (*m/z* 1323, 1307, and 1279), and a terminal residue (*m/z* 892). Analysis of the fraction under fragmenting conditions (Fig. 8) provided a combination of glycosidic cleavage and acyl losses supporting their structure as presented in Table I. The fragment pair at *m/z* 1079/1097 indicates a combined hydroxylmyristic acid elimination and direct loss as a ketene from the *m/z* 1323 ion (the latter fragment, *m/z* 1097, may in addition, be a product of lauroyl loss from the *m/z* 1279 ion). The remaining fragments appear to be related to glycosidic cleavage although the ion, *m/z* 892, was apparent under nonfragmenting conditions. The very abundant ion, *m/z* 892, could originate directly from the *m/z* 1279 parent by glycosidic cleavage since it contains a lauroyl group and was not detected under nonfragmenting conditions. An additional ion, *m/z* 920, suggests it to be the terminal glycosidic fragment from the *m/z* 1307 ion. The ion, *m/z* 1323, $(M - H)^-$, does not possess a lauroyl group (Table I), and a terminal glycosidic cleavage fragment from this ion can be observed, *m/z* 710. The *m/z* 1323 parent ion showed a metastable ion for the hydroxymyristoyl group, with acid elimination in excess over ketene loss. In contrast, the *m/z* 1279 indicated only a minor metastable ion for ketene loss while the *m/z* 1307 ion indicated an absence of any metastable ions. These data suggest a C3 location of the hydroxymyristoyl group in the former ion and the noninvolvement of acyloxy and amide groups in the latter structure (Table I). Following de-*O*-acylation and FAB-MS, only the single ion, *m/z* 871, was obtained.

The fourth fraction, TLC-4, indicated one major triacyl MLA component, *m/z* 1097, and a minor component isobaric with materials described above. A terminal GlcN II ion was also detected, *m/z* 710. Analysis by FAB-MS provided the molecular weight-related ions and fragments shown in Fig. 9.

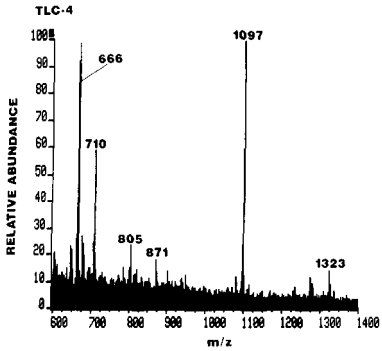


FIG. 9. **Fraction TLC-4 eluted with chloroform/methanol/water (10:5:1) and direct analysis by FAB-MS using triethanolamine:metanitrobenzyl alcohol (1:1) as a FAB matrix.**

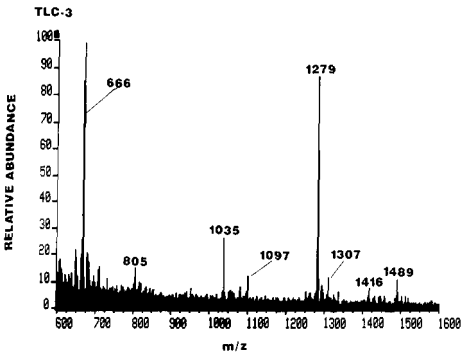


FIG. 10. **Fraction TLC-3 eluted with chloroform/methanol/water (10:5:1) and direct analysis by FAB-MS using triethanolamine:metanitrobenzyl alcohol (1:1) as a FAB matrix.**

The ion at *m/z* 710 increased in relative abundance which reflects the influence of the MNBA matrix. The fragment at *m/z* 871 can be considered a product of hydroxymyristoyl loss from the *m/z* 1097 ion. A check for metastable ions from the *m/z* 1097 parent indicated two minor hydroxymyristoyl fragments (Δ 244/226), with an abundance ratio indicative of a C3 elimination and loss. Upon de-*O*-acylation, only the *m/z* 871 ion was detected. This data indicate the ion *m/z* 710 to be a component of this extract and the structure of the *m/z* 1097 ion to have a C3 hydroxymyristoyl group, (Table I). The other two hydroxymyristoyl groups in this triacyl MLA are located at the C2 and C2′ amide positions; data were provided by de-*O*-acylation. The *m/z* 666 and 805 ions occurred in the TLC blank fraction and a minor ion can be observed at higher mass, the tetraacyl *m/z* 1323 MLA. This probably reflects poor TLC resolution from the neighboring triacyl MLA spot.

Extraction and FAB-MS analysis of the TLC-3 spot gave rise to an abundant higher molecular weight tetraacyl component, *m/z* 1279. De-*O*-acylation of this fraction followed by FAB-MS provided the expected product, *m/z* 872, and a single metastable ion was detected for the elimination of a hydroxymyristic acid group. This major component provided two fragments, *m/z* 1097 and 1035, which may be considered a lauroyl loss (Δ 182) and hydroxymyristoyl elimination (Δ 244) from the parent ion (Fig. 10). Although two isomeric structures may be written for this component, the abundant metastable elimination of a hydroxymyristoyl group suggests its location to be C3′ and not C3, providing the structure in Table I. These data seem to be consistent with an isobaric *m/z* 1279 component detected in TLC-5 which provided a minor metastable fragment. It is surprising to observe the TLC resolution between these isomeric components which seems to indicate a C3 hydroxymyristoyl group location provides the component with greater lipophilicity. Again, the *m/z* 666 ion was detected in high abundance which could represent a glycosidic cleavage fragment of the major component, *m/z* 1279; however, its ubiquitous presence in all fractions and the blank make it impossible to clarify this point. Chromatography with different TLC plates is under investigation. The presence of *m/z* 1489 in this fraction cannot be explained and no isomeric structure can be written for this component. Unfortunately, the concentration of this material was inadequate to evaluate further.

Extraction and FAB-MS analysis of TLC-2 provided several ions in low abundance (Fig. 11). The highest mass ion, *m/z* 1795, could be considered a diphosphoryl hexaacyl Lipid A, analogous to the *m/z* 1715 hexaacyl MLA detected in TLC-7 (Fig. 3, Table I). With the exception of one previously unidentified ion, *m/z* 1335, five other component MLAs (*m/z* 1053, 1079, 1097, 1261, and 1279) were detected in this fraction. These ions were isobaric with components detected in earlier TLC fractions and the *m/z* 1335 fragment could be a hydroxymyristoyl acid elimination product from the *m/z* 1279 component. The monosaccharide ions, *m/z* 710, were detected under fragmenting as well as nonfragmenting conditions. The presence of these ions in the extract could indicate fragments or $(M - H)^-$ components, although it is impossible to be assured of this without having knowledge of the fragmenting behavior of all constituents in the extract. The *m/z* 1079 and 1097 ions have been identified in TLC-5 which were considered to be the combined processes of a hydroxymyristoyl group elimination as the acid, and cleavage as the ketene from the parent ion, *m/z* 1323. This fraction seems to represent a collection of many components in low abundance that appear elsewhere on the TLC plate. No further structural work was carried out with this fraction.

The slowest moving chromatographic spot, TLC-1, indicated two ions of equal abundance when analyzed by FAB-MS under nonfragmenting conditions. An additional ion was detected, *m/z* 853, with the inclusion of MNBA to the FAB matrix. This can be understood as the hydroxymyristic acid elimination fragment originating from the major component, *m/z* 1097 (Fig. 12). When the fraction was de-*O*-acylated and reanalyzed, two ions were detected: the *m/z* 871 ion and the glycosidic fragment, *m/z* 440. This would indicate two hydroxymyristoyl groups are amide-linked in the *m/z* 1097 component, and the remaining problem is to account for the third hydroxymyristoyl group and determine its location. This was resolved by MIKES analysis which showed a major fragment at *m/z* 853 from the focused parent *m/z* 1097. This acid elimination strongly suggests the third hydroxymyristoyl group to be located at the C3′ position. An isobaric component to this *m/z* 1097 ion was detected in TLC-4 and the structure

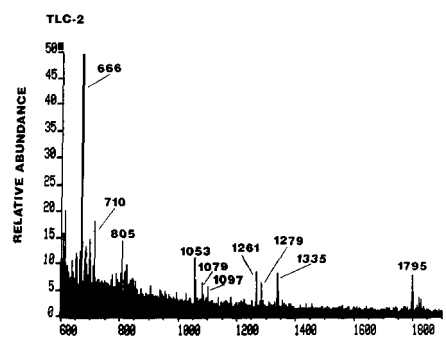


FIG. 11. **Fraction TLC-2 eluted with chloroform/methanol/water (10:5:1) and direct analysis by FAB-MS using triethanolamine:metanitrobenzyl alcohol (1:1) as a FAB matrix.**

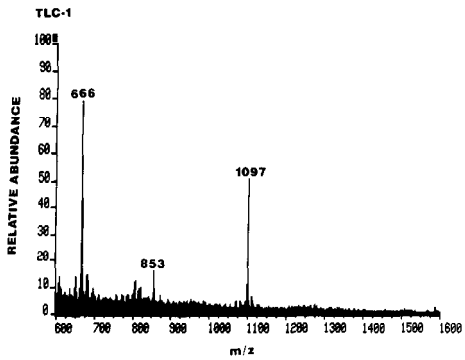


FIG. 12. **Fraction TLC-1, eluted with chloroform/methanol/water (10:5:1) and direct analysis by FAB-MS using triethanolamine:metanitrobenzyl alcohol (1:1) as a FAB matrix.**

of this ion was supported by an absence of any metastable elimination ion.

DISCUSSION

Since the introduction of soft ionization, mass spectral analysis has provided major contributions to the structural understanding of Lipid A samples. The success of this approach can be attributed to a combination of molecular weight measurement of purified components and specific fragments that define structural detail. Two ionization techniques have been reported, fast atom bombardment (10, 11, 22, 23) and laser desorption (24–28). These techniques have been coupled with magnetic sector and time of flight analyzers for positive ion focusing and mass measurement. For the development of molecular weight-related ions, both desorption methods are highly dependent on adduct formation with added or indigious metal salts. With samples isolated from a biological matrix and containing a mutliplicity of cations, this can cause considerable signal dispersal and be compounded further with the very facile formation of pyranoxonium ions. This dispersal of parent and fragment signals greatly increases the probability of unresolvable isobaric events. Since the first introduction of these approaches, and with a better understanding of Lipid A structures, improvements has been made in sample preparation and selecting instrumental parameters that maximize structural detail.

In this structural study of Lipid A samples using FAB-MS, we have found it advantageous to use two different matricies: one for a determination of sample composition in mixtures, *e.g.* to maximize molecular weight-related ions, and another that provides a predominance of fragmentation to study molecular detail. Using this latter matrix, in combination with negative ion extraction, yields a more equal abundance of molecular weight-related and fragment ions, and, in addition, negative ion extraction avoids signal dispersal due to cation adduct formation. Under these conditions the fragments are simple and easily understood processes and (with the exception of anomeric configuration) provide adequate information for complete characterization. The major ions are phosphoryl anions with fragments generated by single or multiple losses of ketene, acid, and GlcN I. In selected cases where fragments are unable to clarify component structure, or where isomeric structures are possible, we have utilized parent ion focusing and the detection of metastable ion products. Thus for the $(M - H)^-$ components (*m/z* 1279 and 1097), found in two TLC fractions, MIKES was able to position the hydroxymyristoyl group to a C3′ position in one structure and C3 in the other. This approach has considerable advantage in complex mixtures by allowing a structural evaluation of each focused parent ion. The data collected from some fractions are difficult to interpret, especially for GlcN II fragments. The detection of the ion *m/z* 666 in most fractions, and the blank, makes it impossible to consider this a GlcN II-related component or fragment. The complexity of TLC-2, both in diversity of structures and number of components detected could not be resolved. Clearly, this structural study might have gained from by on-line chromatographic-MS analysis.

The assignment of structure to many of the $(M - H)^-$ ions in Table I is based on the following assumptions: (*a*) absolute enzyme specificity in the biosynthesis of these MLA products; (*b*) a structural understanding of the heptaacyl MLA from *S. minnesota* (16); (*c*) the chemical lability and product degradation during acid hydrolysis (19, 29, 30); and (*d*) the loss of defined residues from specific sites during FAB-MS analysis. The observed fragments in negative and positive ion FAB-MS, chemical de-*O*-acylation, and the metastable ion spectra combine to provide acyl position for each component. Two components, *m/z* 1279 and 1097, were each detected in different TLC fractions. The isomeric *m/z* 1279 was detected in TLC-5 and TLC-3, while *m/z* 1079 was resolved into fractions TLC-4 and TLC-1. The structures of each isomeric material were assigned by MIKES analysis taking advantage of the hydroxymyristoyl metastable character (acid elimination) when located at the C3′ position. It is of some interest when considering the isomers of both structures (*m/z* 1279 and 1097), that a hydroxymyristoyl group located in the C3 *versus* the C3′ position imparts to the molecule a greater lipophilic character. This acid elimination over a ketene-type loss may be attributed to the anchimeric assistance provided by the adjacent phosphate group. When collision gas was used during MIKES analysis no qualitative difference in the spectra was observed, so to improve mass accuracy these studies were carried out in the absence of a collision gas.

The *S. minnesota* Re595 monophosphoryl Lipid A analogs present in these commercial preparations raises two questions. First, what factors contribute to the heterogeneity of Lipid A: incomplete biosynthesis of the heptaacyl MLA, acid degradation during MLA preparation, or both? Second, do the different MLA analogs in Lipid A preparations exhibit different biological activities? A probable explanation of heterogeneity may be that it is the result of acid degradation during the hydrolytic conversion of the diphosphoryl to the monophosphoryl Lipid A analogs (19, 29, 30). Regardless of their origin, a structure-function analysis of these homologs offers a unique opportunity to evaluate and relate specific biological properties. To the degree that some of these analogs may be artifactual (acid degradation products) they could lead to unique activities or better focus specific functions not available with the study of natural products. Such an analysis is presented in the accompanying paper (17).

Addendum—More recent studies have resulted in the separation of 12 Lipid-A homologs using supercritical fluids as a mobile phase (supercritical fluid chromatography). Structural characterization of these materials by on-line supercritical fluid chromatography-MS is currently underway.

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