papers on methodology

α-D-Glucopyranosyl-, D-alanyl- and L-lysylcardiolipin from gram-positive bacteria: analysis by fast atom bombardment mass spectrometry

J. Peter-Katalinic[1,*] and W. Fischer[2,†]

Institut für Physiologische Chemie,* Universität Bonn, Nussallee 11, D-53115 Bonn, Germany; and Institut für Biochemie,† Med. Fak., Universität Erlangen-Nürnberg, Fahrstrasse 17, D-91054 Erlangen, Germany

Abstract **Cardiolipin species substituted on O2 of the middle glycerol moiety with α-D-glucopyranosyl, D-alanyl and L-lysyl residues were isolated from different gram-positive bacteria. There respective structures were elucidated by positive and negative mode fast atom bombardment mass spectrometry. The structural heterogeneity due to different fatty acid combinations was documented by up to seven molecular ions. General structural features were derived from diagnostic fragment ions, generated by single cleavage at the phosphodiester moieties in both positive and negative ion mode. A diagnostically important fragment ion for D-alanylcardiolipin was observed in the positive ion mode. It arose from double cleavage of the phosphodiester moieties yielding [NaH(Na) PO_4-CH_2·CH(OCO·$CHNH_2$·CH_3) CH_2^+]$^+$. The fatty acid combinations in the phosphatidyl and diacylglycerol ions make it possible to recognize whether saturated and unsaturated fatty acids were selectively or randomly distributed on the two positions of the glycerol moieties. Molecular structures of cardiolipins, derived from mass spectrometric experiments, are in full agreement with those, elucidated by classical chemical analyses.—**Peter-Katalinic, J., and W. Fischer. **α-D-Glucopyranosyl-, D-alanyl- and L-lysylcardiolipin from gram-positive bacteria: analysis by fast atom bombardment mass spectrometry. *J. Lipid. Res.* 1998.** 39: **2286–2292.**

Supplementary key words cardiolipin • d-alanylcardiolipin • d-glucopyranosylcardiolipin • L-lysylcardiolipin • listeria • streptococci (group B) • vagococci • FAB MS

Phosphatidylglycerol (PtdGro) and bisphosphatidylglycerol (Ptd_2Gro, cardiolipin) are wide-spread negatively charged lipid components in the cytoplasmic membranes of gram-positive and gram-negative bacteria (see reviews by O'Leary and Wilkinson (1) and Wilkinson (2)). Since the discovery of alanylphosphatidylglycerol in *Clostridium welchii* by MacFarlane in 1962 (3), aminoacylester of PtdGro have been detected in many gram-positive (1, 4, 5), but apparently not in gram-negative bacteria (2). Frequently observed ligands were d-alanine and l-lysine. Glycosylated PtdGro has so far only been found in certain strains of *Bacillus megaterium* in the form of 2-amino-2-deoxy-β-d-glucopyranosyl derivatives (6, 7). Later on, a substituted cardiolipin was detected in several strains of *Streptococcus* group B and identified as α-d-glucopyranosylPtd_2Gro (8). In group B *Streptococcus*, it amounts to approximately 18% of the lipid phosphorus and was as a minor component found in *Vagococcus fluvialis* (9). From *V. fluvialis*, d-alanylPtd_2Gro was isolated and characterized. It was shown to occur in all strains tested and contributed up to 38% of the lipid phosphorus (9). l-LysylPtd_2Gro, another cardiolipin derivative, occurs in listeria and was detected in each of the four species tested: *Listeria monocytogenes*, *Listeria innocua*, *Listeria seeligeri*, and *Listeria welshimeri*. It contributes between 11 and 26 mol percent to the polar lipids (W. Fischer and K. Leopold, unpublished data).

In the present report we describe negative and positive ion fast atom bombardment mass spectrometry (FAB MS) of the three substituted cardiolipins. The fragmentation pattern of phospholipids has been established by previous workers using phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, and phosphatidylinositol (10–14). Cardiolipin was studied by negative ion MS/MS. The sample used contained only one fatty acid species (18:2) and accordingly showed a single molecular ion (15).

MATERIALS AND METHODS

Lipids

d-GlcPtd_2Gro was isolated from *Streptococcus* group B type Ia, Kiel 090 (8), d-AlaPtd_2Gro from *V. fluvialis*, NCDO 2497 (9), and l-

Abbreviations: PtdGro, Phosphatidylglycerol; Ptd_2Gro, cardiolipin, bisphosphatidylglycerol; AlaPtd_2Gro, d-alanylcardiolipin; GlcPtd_2Gro, d-glucosylcardiolipin; LysPtd_2Gro, l-lysylcardiolipin; FAB-MS, fast atom bombardment-mass spectrometry

[1]Present address: Inst. Medizinische Physik und Biophysik, Universität Münster, Germany.

[2]To whom correspondence should be addressed.

LysPtd$_2$Gro from *L. welshimeri*, SLCC 5334 (W. Fischer and K. Leopold, unpublished data). Briefly, lipids were extracted from mechanically disintegrated bacteria by a modified acidic Bligh Dyer procedure, fractionated by column chromatography on DEAE cellulose, and finally individual lipids were purified by chromatography on Iatrobeads (Iatron Laboratories, Tokyo) and/or preparative TLC. As far as possible, the pH was kept at 4.7 in order to avoid base-catalyzed hydrolysis of the labile O-aminoacyl ester bonds (16). The structures of the cardiolipin derivatives, including the characterization of the fatty acid composition, were established by chemical and enzymatic procedures (8, 9; W. Fischer and K. Leopold, unpublished data). The purified lipids were stored at −20°C in $CHCl_3$–MeOH 2:1 (by vol; slightly acidified with acetic acid).

Fast atom bombardment mass spectrometry

FAB MS was performed on a ZAB-HF mass spectrometer (VG Analytical, Manchester, UK) at the University of Bonn essentially as described (17). The lipids were dissolved in $CHCl_3$–MeOH 2:1 (by vol) and desorbed from the matrix thioglycerol (positive ion mode) or triethanolamine (negative ion mode) with xenon as bombarding gas. The mass spectra were acquired as single scans in the upscan mode on a AMD DP10 data system, fitted with SAMII (KWS) hardware and SUSY software (AMD Intectra, Beckeln, Germany). Mass values were obtained after calibration with CsI and represent nominal mass numbers.

RESULTS AND DISCUSSION

α-d-Glucopyranosylcardiolipin (GlcPtd$_2$Gro), l-alanylcardiolipin (AlaPtd$_2$Gro), l-lysylcardiolipin (LysPtd$_2$Gro), and the cardiolipin (Ptd$_2$Gro) coextracted with the LysPtd$_2$Gro were analyzed by negative and positive ion

TABLE 1. Molecular and fragment ions in negative ion FAB MS

	GlcPtd$_2$Gro		AlaPtd$_2$Gro		Ptd$_2$Gro		LysPtd$_2$Gro	
Molecular Ions	[M–H$^+$]$^-$[a]		[M–H$^+$]$^-$		[M–H$^+$]$^-$		[M–H$^+$]$^-$	
	m/z	Fatty acids[b]	*m/z*	Fatty acids[b]	*m/z*	Fatty acids[c]	*m/z*	Fatty acids[c]
	1593	18/18/18/16 2Δ	1474	18/18/16/16 2Δ	1379	17/17/17/15	1507	17/17/17/15
	1565	18/18/16/16 2Δ	1448	18/16/16/16 1Δ	1365	17/17/16/15		
	1537	18/16/16/16 2Δ	1446	18/16/16/16 2Δ	1351	17/17/15/15	1479	17/17/15/15
	1511	18/16/16/14 1Δ		18/18/16/14 2Δ	1335	17/x[d]/15/15	1463	17/x[d]/15/15
					1323	17/15/15/15	1451	17/15/15/15
					1307	x[d]/15/15/15	1435	x[d]/15/15/15
					1295	15/15/15/15		
Fragment Ions	*m/z*		*m/z*		*m/z*		*m/z*	
a	1327	1565–238 (16:0)			1127	1379–252 (17:0)		
	1301	1565–264 (18:1)				1365–238 (16:0)		
						1351–224 (15:0)		
					1113	1365–252 (17:0)		
					1099	1351–252 (17:0)		
						1323–224 (15:0)		
b	989	1566–577 (18:1, 16:0)			801	1352–551 (15:0, 17:0)		
		(18:0, 16:1)						
c			699	(18:1, 18:1)	661	(17:0, 17:0)		
	673	(18:1, 16:0), (18:0, 16:1)	673	(18:1, 16:0)	647	(17:0, 15:0)	647	(17:0, 15:0)
			671	(18:1, 16:1)	631	(16:1, 15:0)	631	(16:1, 15:0)
	645	(16:0, 16:1)	645	(16:0, 16:1)	619	(15:0, 15:0)	619	(15:0, 15:0)
			643	(16:1, 16:1)	603	(14:1, 15:0)	603	(14:1, 15:0)
d	435	673–238 (16:0)	435	699–264 (18:1)	423	647–224 (15:0)	423	647–224 (15:0)
	409	673–264 (18:1)		673–238 (16:0)				
		645–236 (16:1)		671–236 (16:1)				
				645–210 (14:0)				
e			417	699–282 (18:1)	389	631–242 (15:0)	389	631–242 (15:0)
				673–256 (16:0)				
				671–254 (16:1)				
				645–228 (14:0)				
f′,f[e]	283	18:0 [16.4]	283	18:0 [8.6]	269	17:0 [26.8]	269	17:0 [24.7]
	281	18:1 [25.2]	281	18:1 [38.2]	253	16:1 [<0.5]	255	16:0 [9.4]
	255	16:0 [32.8]	255	16:0 [27.2]	255	16:0 [6.3]	253	16:1 [<0.5]
	253	16:1 [16.4]	253	16:1 [16.2]	241	15:0 [63.2]	241	15:0 [59.1]
	227	14:0 [3.8]	227	14:0 [9.7]		14:0 [1.6]		14:0 [2.6]
g,h	97,79	$H_2PO_4^-$,PO_3^-	97,79	$H_2PO_4^-$,PO_3^-	79	PO_3-	97,79	$H_2PO_4^-$,PO_3^-
Not assigned	1481, 715, 107		938, 727, 153, 137		841, 705, 689, 107		709, 609, 269, 153	

[a] Higher values were at *m/z* 1615, 1587, 1559, corresponding to [M–2 H$^+$ + Na$^+$]$^-$.

[b] Straight-chain saturated and monounsaturated fatty acids; Δ denotes number of monoenic acids; their assignment to C18 or C16 would be arbitrary.

[c] Anteiso- and iso-branched fatty acids: a-17:0 (14-methylhexadecanoate); i-17:0 (15-methylhexadecanoate); a-15:0 (12-methyltetradecanoate); i-15:0 (13-methyltetradecanoate). In both lipids a-15:0, i-15:0, a-17:0, i-17;0 contributed approximately 50, 10, 22, and 4 mol%, respectively.

[d] "x," corresponds formally to 16:1.

[e] Values in square brackets, relative abundance, determined by GLC.

TABLE 2. Molecular and fragment ions in positive ion FAB MS

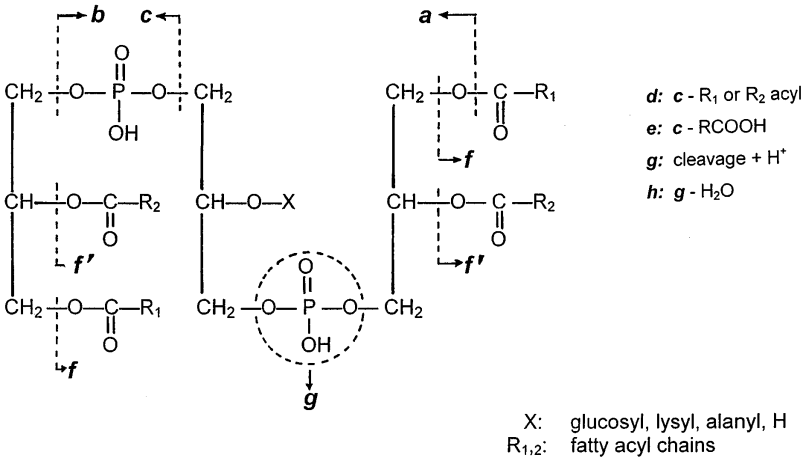
	$GlcPtd_2Gro$		$AlaPtd_2Gro$		$LysPtd_2Gro$	
Molecular Ions	$[M–2H^+ + 3Na^+]^+$		$[M–2H^+ + 3Na^+]^+$		$[M+H^+]^+$ / + 92[b]	
	m/z	Fatty acids[a]	*m/z*	Fatty acids[a]	*m/z*	Fatty acids[a]
	1661	18/18/18/16 2Δ	1542	18/18/16/16 2Δ	1481/1573	17/17/15/15
	1633	18/18/16/16 2Δ	1514	18/16/16/16 2Δ	1467/1559	17/15/15/16:0
	1605	18/16/16/16 2Δ			1453/1545	17/15/15/15
Fragment Ions	*m/z*		*m/z*		*m/z*	
i, i′					1257/1349	1481–224 (15:0)/+92
					1229/1321	1481–252 (17:0)/+92
					931	1481–550 (15:0, 17:0)
k					903	1453–550 (15:0, 17:0)
	719	(16:0, 18:1)	719	(16:0, 18:1)		
l	691	(16:1, 16:0)	717	(16:1, 18:1)		
		(18:1, 14:0)	691	(16:1, 16:0)		
				(18:1, 14:0)		
			689	(16:1, 16:1)		
m	463	719–256 (16:0)	463	719–256 (16:0)		
		691–228 (14:0)		691–228 (14:0)		
	437	719–282 (18:1)	437	719–282 (18:1)		
		691–254 (16:1)		691–252 (16:1)		
n	577/599 (18:1, 16:0)/+Na		577	(18:1, 16:0)	551/643	(15:0, 17:0)/+92
	549	(16:0, 16:0)	549	(16:1, 16:0)	537/629	(15:0, 16:0)/+92
		(18:1, 14:0)		(18:1, 14:0)	523/615	(15:0, 15:0)/+92
o	361	599–238 (16:0)			327	551–224 (15:0)
	339	577–238 (16:0)			299	551–252 (17:0)
						537–238 (16:0)
Not assigned	971[c], 785, 279, 199, 133, 125		759, 350, 125		345	

[a]For structure of fatty acids, their relative abundance and explanation of Δ, see Table 1.
[b]For discussioin of +92, see text.
[c]*m/z* 971 may be explained by $[M - {}^{-}OCH_2 \cdot CHOCOR \cdot CH_2OCOR]$ (16:0, 18:0).

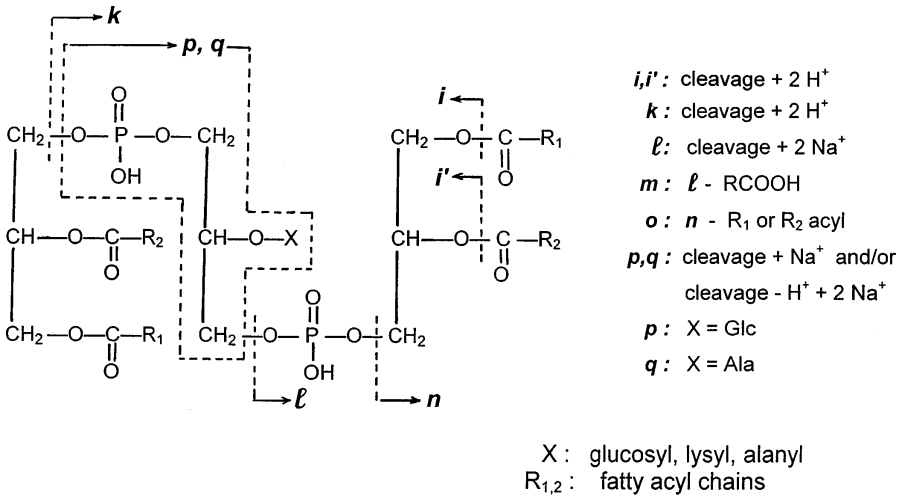
FAB mass spectrometry. Molecular ions and fragment ions are summarized in **Table 1** and **Table 2**, the underlying fragmentations are shown in **Scheme 1** and **Scheme 2**. The constituent fatty acids were identified in the negative ion mode as carboxylate ions (***f′***, ***f***) and are listed in Table 1 together with their relative abundance which was determined by GLC (9, 18; W. Fischer, and K. Leopold, unpublished data). The ions ***g***, ***h***, which are observed at *m/z* 97 and 79 in negative ion mode of all samples (Table 1), belong to the double cleavage ions (Scheme 1) and are indicative of the phosphate groups.

Molecular ions

Negative and positive ion FAB mass spectra of $GlcPtd_2Gro$, $AlaPtd_2Gro$, and $LysPtd_2Gro$ are shown in **Figs. 1**, **2**, **3**, and **4**. All lipids were composed of several molecular species due to various fatty acids combinations. The peaks of the molecular ions were of higher intensity in negative ion mode and the molecular weights could be derived from them directly. For instance, $AlaPtd_2Gro$ displayed in negative ion mode three molecular ions $[M + H^+]^-$ at *m/z* 1474, 1448, and 1446 (Table 1, Fig. 2A); in positive ion mode only the molecular ion $[M - H^+]^+$ of



Scheme 1. Fragment ions formed in negative ion FAB MS of non-substituted and substituted cardiolipin**.** For the sake of clarity, the fragmentation sites for ***a***, ***b***, ***c***, and ***g*** are indicated only on one of the two phosphatidyl residues.



Scheme 2. Fragment ions formed in positive ion FAB MS of substituted cardiolipin. For the sake of clarity, the fragmentation sites for ***i***, ***k***, ***l***, ***n***, and ***p***, ***q*** are indicated only on one of the two phosphatidyl residues.

the most abundant species appeared at *m/z* 1476. It was accompanied by $[M + Na^+]^+$ at *m/z* 1498 (not shown) and, after addition of sodium acetate to the matrix, two species were recognized as $[M - 2\ H^+ + 3\ Na^+]^+$ at *m/z* 1542 and 1514 (Table 2, Fig. 2B). Likewise, in positive ion mode $GlcPtd_2Gro$ provided trisodiated molecular ions $[M - 2\ H^+ + 3\ Na^+]^+$ at *m/z* 1661, 1633, 1605 (Table 2). In negative ion mode four molecular ions $[M - H^+]^-$ were detected at *m/z* 1593, 1565, 1537, 1511, which were accompanied by a series $[M - 2\ H^+ + Na^+]^-$ of lower intensity at *m/z* 1615, 1587, 1559 (not shown).

Ptd_2Gro and $LysPtd_2Gro$, isolated from the same lipid

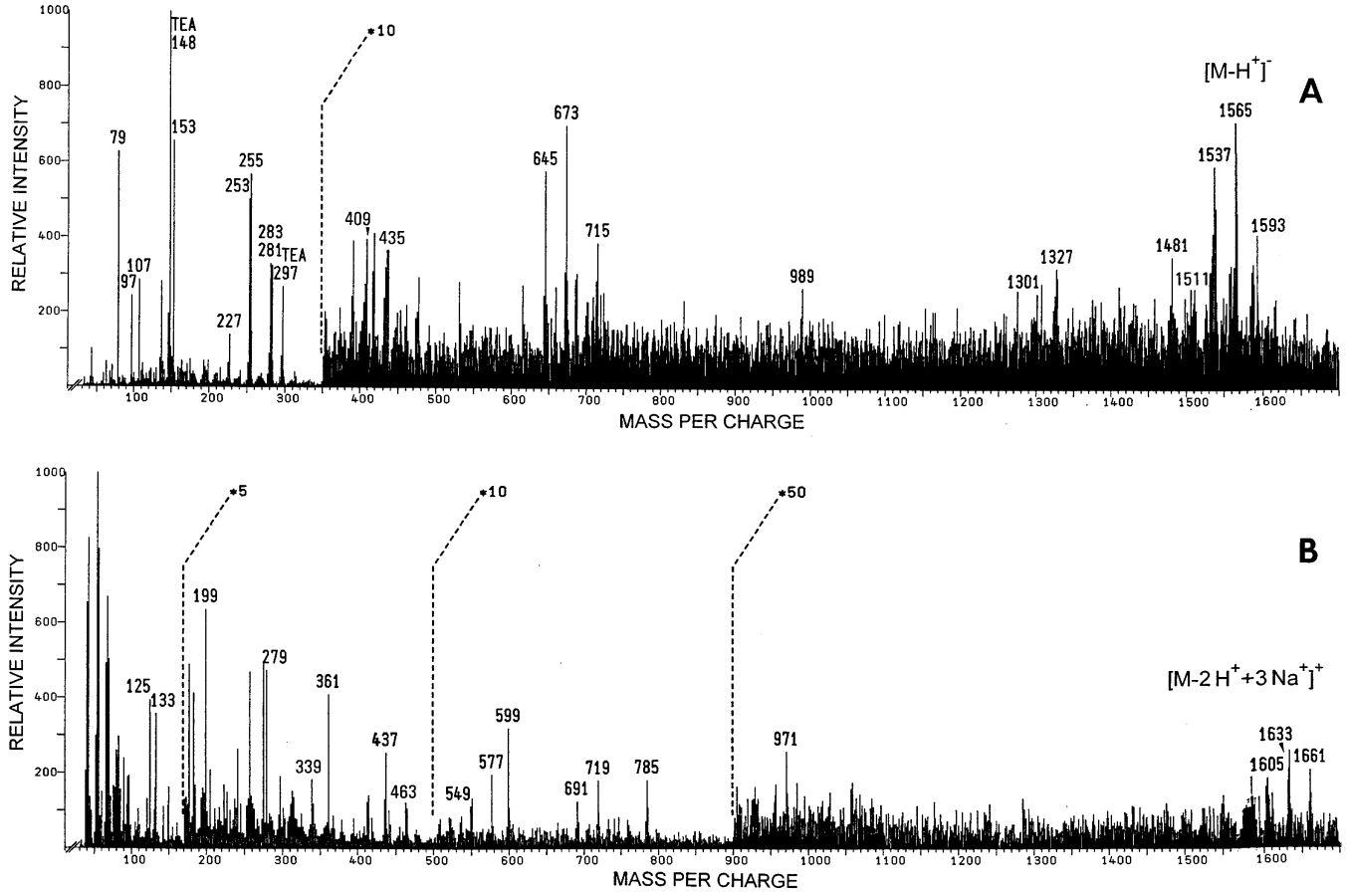


Fig. 1. FAB-mass spectrum of d-glucopyranosyl cardiolipin from *Streptococcus* B in negative (A) and positive ion mode (B).

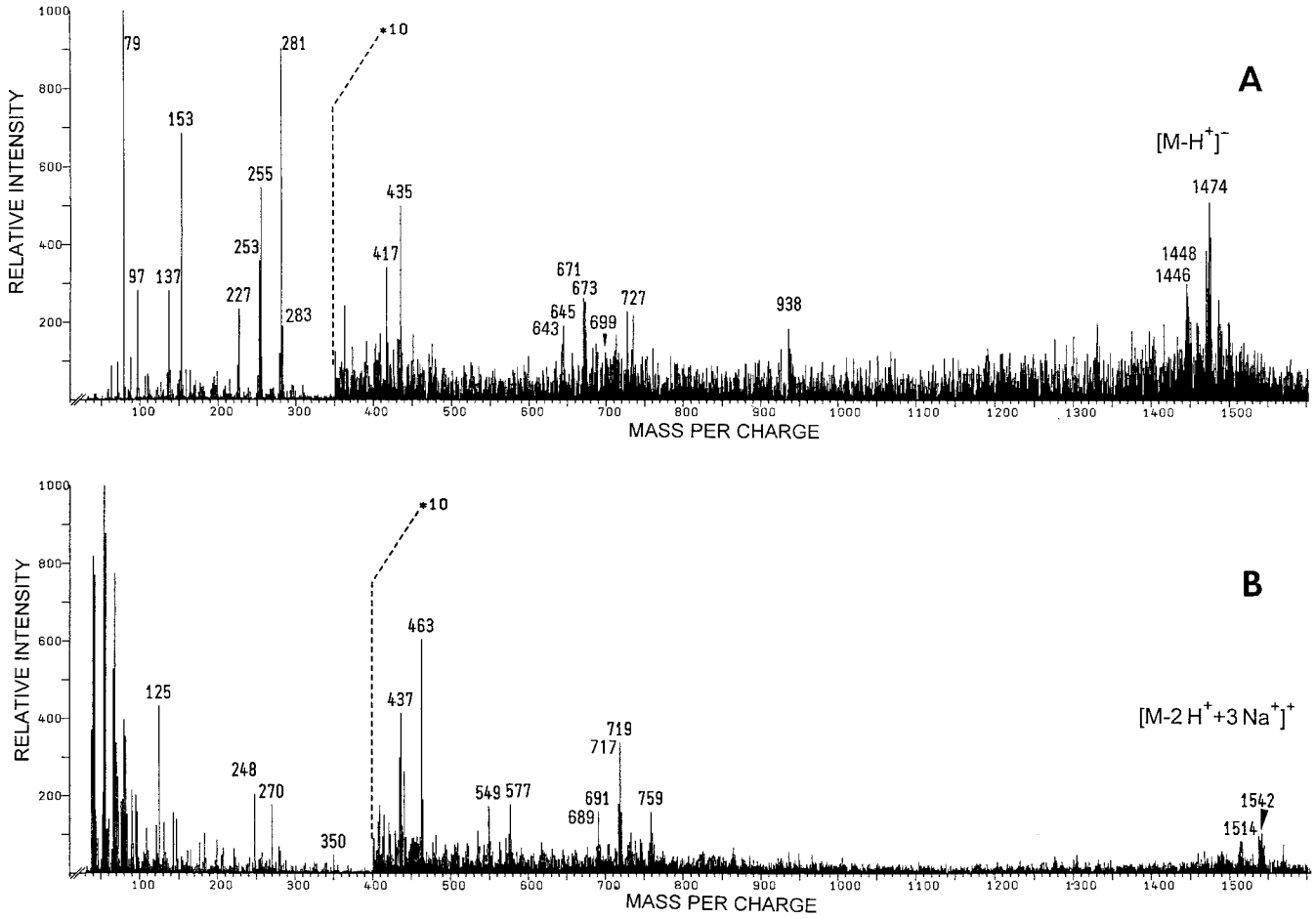


Fig. 2. FAB-mass spectrum of d-alanylcardiolipin from *Vagococcus fluvialis* in negative (A) and positive ion mode (B).

extract of *L. welshimeri*, possessed a very similar fatty acid composition. This is documented by identical carboxylate ions and the relative abundance of the constituent fatty acids, both shown in Table 1. In negative ion mode the two lipids showed five molecular ions $[M - H^+]^-$ that differed from each other by 128 amu (Table 1), consistent with the chemically established lysyl substituent (W. Fischer, and K. Leopold, unpublished data).

In positive ion FAB MS, the molecular ions $[M + H^+]^+$ of $LysPtd_2Gro$ were accompanied by a set of ions showing an increment of 92 amu (Fig. 4B) and the fragment ions ***i***, ***i′*** and ***n*** also displayed analogs with this increment (Table 2). A covalently linked constituent, which might have been overlooked on chemical analysis, is unlikely because no adequate molecular and fragment ions were found in negative FAB MS. A complexation with glycerol (molecular weight 92) can also be excluded because glycerol was not added to the analyte/matrix mixture. As the other substituted cardiolipins did not show this feature, one might suspect that the increment of 92 amu was accomplished by complexation with a degradation product of $LysPtd_2Gro$. An intermolecular transfer of choline and its degradation products (loss of H_2O and loss of $H_2O + CH_2$) was reported previously to occur in positive ion FAB MS of phosphatidylcholine (11).

Fragment ions

The fragmentations, observed in negative and positive ion mode, are summarized in Schemes 1 and 2. Fragment ***a*** $[M - H^+ -$ fatty acyl chain$]^-$ and fragment ***b*** $[M - {}^+CH_2{\cdot}CH(OCOR) \cdot CH_2OCOR]^-$ were observed in the negative ion FAB MS of $GlcPtd_2Gro$ and Ptd_2Gro (Table 1). In positive ion mode, analogous fragments ***i***, ***i′*** $[M + 2\ H^+ -$ fatty acyl chain$]^+$ and ***k*** $[M + 2\ H^+ - {}^+CH_2{\cdot}CH(OCOR){\cdot}CH_2OCOR]^+$ were formed from $LysPtd_2Gro$.

In negative ion mode, the diagnostically important ions ***c*** are created by cleavage between the oxygen of the phosphatidyl residue and the CH_2 group of the middle glycerol moiety (Scheme 1). Phosphatidyl ions ***c*** were released from all four compounds (Table 1). From fragment ***c***, two kinds of daughter ions were derived (Table 1): ion ***d*** arose by loss of a single acyl residue; ion **e** by release of a neutral fatty acid molecule (Scheme 1).

As shown in Scheme 2, phosphatidyl residues were also released in positive ion mode from $GlcPtd_2Gro$ and $AlaPtd_2Gro$, but the resulting ions ***l*** at *m/z* 691, 719 were upshifted by 46 amu due to the addition of 2 Na^+ (Table 2). Loss of a neutral fatty acid molecule from ***l*** produced the daughter ions ***m*** at *m/z* 437, 463. In addition to phosphatidyl ions ***l***, in positive ion mode diacylglycerol ions ***n***

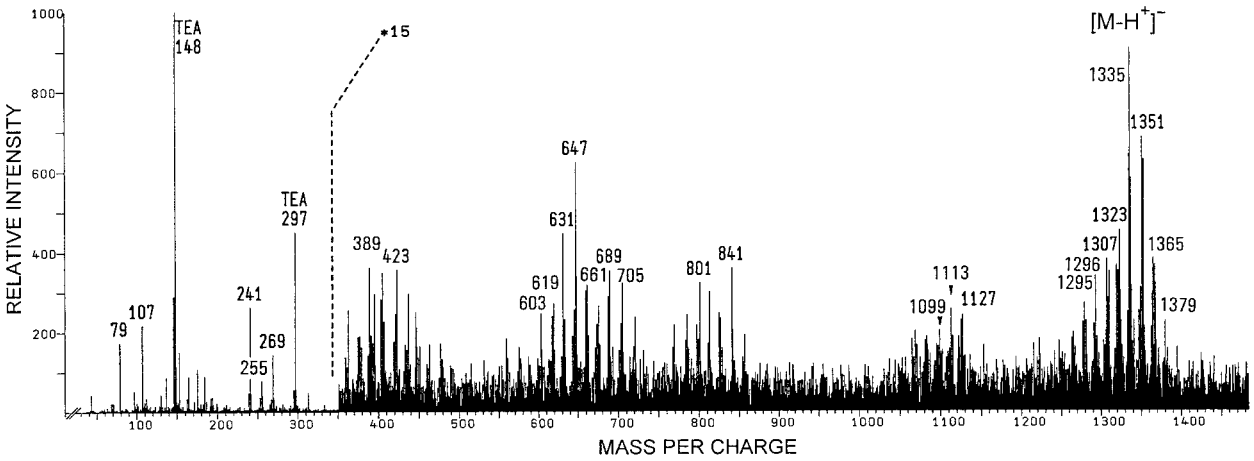


Fig. 3. FAB-mass spectrum of cardiolipin from *Listeria welshimeri* in negative ion mode.

of *m/z* 577 and 549 were generated. Subsequent loss of a single fatty acyl chain (16:0) led to ions ***o*** at *m/z* 361 and 339 (Scheme 2, Table 2).

From the positive ion FAB mass spectrum of Lys Ptd_2Gro, phosphatidyl ions ***l*** were absent (Fig.3B). Diacylglycerol ions ***n*** appeared at *m/z* 551, 537, and 523, and ions ***o*** of *m/z* 327 and 299 were derived from them by loss of a single fatty acyl chain.

Ions, diagnostic for the substituents

The positive ion mode proved diagnostically particularly important. Double cleavage released the internal phosphoglycerol to which the respective substituent may remain attached (Scheme 2).

The most abundant ion of $AlaPtd_2Gro$ was ion ***q*** (Fig. 2B) which proves the substitution of the middle glycerol moiety with alanine:

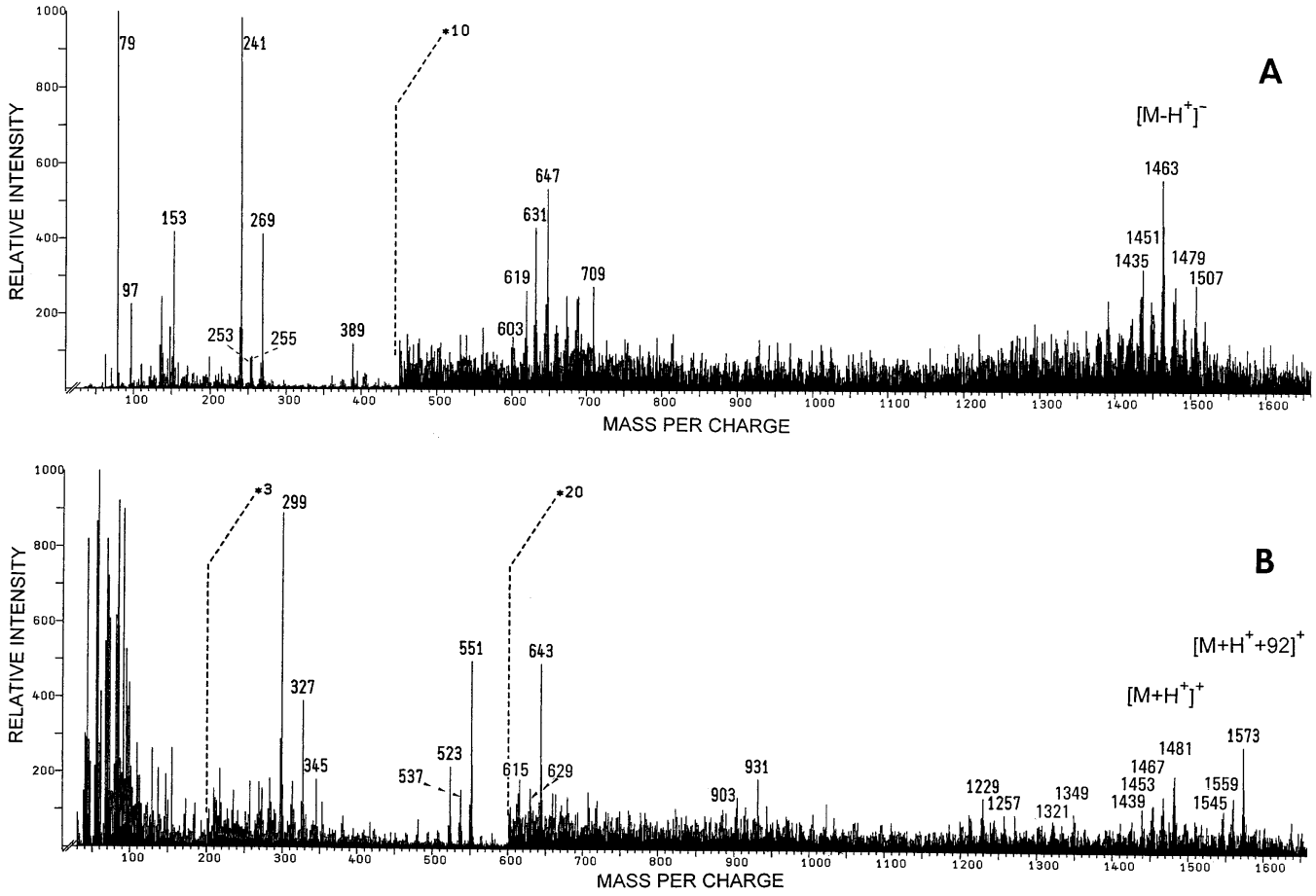
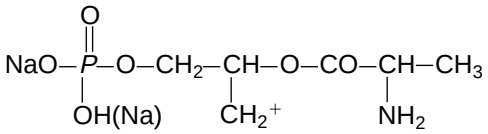
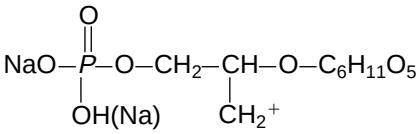


Fig. 4. FAB-mass spectrum of l-lysylcardiolipin from *Listeria welshimeri* in negative (A) and positive ion mode (B).



q: m/z 248 (270)

In the case of $GlcPtd_2Gro$, the situation is less clear. The glucosylglyceromonosodium phosphate (ion ***p***) provides an ion at *m/z* 339, the disodium form an ion at *m/z* 361.



p: m/z 339 (361)

As shown in Table 2, the fragments at *m/z* 339 and 361 also correspond to the monoacylglycerol ion ***o*** and its monosodium form, containing octadecenoic acid.

In the positive ion FAB MS of $LysPtd_2Gro$ an ion of *m/z* 305 corresponding to the monosodiated lysylglycerophosphate ion was not present. The identity of the ion at *m/z* 327 as its disodiated form (Fig. 3B) is not unambigous because ion ***o***, resulting from loss of a single acyl chain from fragment ***n***, has the same mass/charge ratio (Table 2).

Ions, diagnostic for fatty acid distribution

The phosphatidyl ions ***c***, observed in negative ion mode, provide evidence for the fatty acid distribution on the two positions of the glycerol moieties. $GlcPtd_2Gro$ yielded two species at *m/z* 673 and 645, each containing one saturated and one unsaturated fatty acid. This is in accord with earlier results of enzymatically performed fatty acid distribution analysis which showed hexadecanoate and octadecanoate preferentially linked to O1 of the glycerol moiety, hexadecenoate and octadecenoate to O2 (18). By contrast, the phosphatidyl ions ***c*** of $AlaPtd_2Gro$ comprised five species at *m/z* 699, 673, 671, 645, and 643, with substantial abundance of the species at *m/z* 671 (Fig. 2B). Only the ions at *m/z* 673 and 645 contained the combination of saturated and unsaturated fatty acid, in the other species two unsaturated fatty acids were combined (Table 1). In positive ion mode, species containing two unsaturated fatty acids were represented by the ions of *m/z* 717 and 689 (Table 2). These data indicate, that $AlaPtd_2Gro$, in contrast to $GlcPtd_2Gro$, displays a more random distribution of fatty acids.

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