Supplements

Analysis of aminoacylated cardiolipins and phosphatidylglycerols from bacteria

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2. Experimental methods

2.1. Chemicals and Standards

The L and D amino acid standards were obtained from Sigma–Aldrich (now Merck, Prague, Czech Republic), i.e. L-threonine, D-threonine, L-allo-threonine, D-allo-threonine, L-alanine, D-alanine, L-arginine, D-arginine, L-isoleucine, D-isoleucine, L-allo-isoleucine, D-allo-isoleucine, L-leucine, D-leucine, L-lysine, D-lysine, cardiolipin (CL), free fatty acid (FFA), phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylserine (PS), acetonitrile (ACN), and methanol (MeOH) of LC–MS grade. *N*-(4-nitrophenoxycarbonyl)-l-phenylalanine 2-methoxyethyl ester ((S)-NIFE) was purchased from Cayman Europe OÜ (Tallinn, Estonia).

2.2. Strain and cultivation.

Geobacillus stearothermophilus (CCM 3537) from CCM collection (Brno, Czech Republic). The culture of bacterium was grown in minimal M9 medium (0.4% $(NH_4)_2SO_4$, 1.36% KH_2PO_4, 0.3% NaOH, 0.2% $MgSO_4 \times 7H_2O$, 0.02% $CaCl_2 \times 6H_2O$, 0.01% $FeSO_4 \times 7H_2O$, pH 7.2) supplemented with 10 g/L casein hydrolysate. Cultures were incubated in 100 mL of a medium on an orbital shaker (190 rpm) for 48 h and at 65 °C.

Alicyclobacillus acidoterrestris CCM 4660 was obtained from the Czech Collection of Microorganisms (Brno, Czech Republic) and it was cultivated in an alicyclobacillus medium [1] containing (g/L): yeast extract 6.0, glucose 5.0, $CaCl_2 \cdot \times 2H_2O$ 0.25, $MgSO_4 \cdot \times 7H_2O$ 0.5, $(NH_4)_2SO_4$ 0.2, KH_2PO_4 3.0 and 1 ml/L of trace element solution in g/L: $ZnSO_4 \cdot \times 7H_2O$ 0.1, $MnCl_2 \cdot \times 4H_2O$ 0.03, H_3BO_3 0.3, $CuCl_2 \cdot \times 6H_2O$ 0.2, $CaCl_2 \cdot \times 2H_2O$ 0.01, $NiCl_2 \cdot \times 6H_2O$ 0.02, $Na_2MoO_4 \cdot \times 2H_2O$ 0.03; pH was adjusted to 4. Batch cultivation was carried out under aerobic conditions in 500 mL Erlenmeyer flasks filled with 200 mL of medium. Erlenmeyer flasks were placed in a temperature-controlled shaking device [1]. Cultivation proceeded for 24 h, at 45 °C and 200 r.p.m. After cultivation, the biomass was concentrated by centrifugation (10 000 r.p.m.) and lyophilized.

Bacillus licheniformis CCM 2205, medium (g/L): KH_2PO_4, 1.36; $(NH_4)_2SO_4$, 1.0; $MgSO_4 \times 7H_2O$, 0.2; FeSO4, 0.01; NaCl, 2.0; yeast extract, 1.0. The medium was supplemented 1 % w/v glucose. *B. licheniformis* was cultured in 500 mL Erlenmeyer flasks containing 150 mL of medium at pH 6.5 and culture was incubated in a shaker at 200 rpm at 50 °C for 24 h [2].

References

[1] Siristova L, Melzoch K, Rezanka T. Fatty acids, unusual glycophospholipids and DNA analyses of thermophilic bacteria isolated from hot springs. Extremophiles. 2009;13(1):101–109. doi:10.1007/s00792-008-0202-6

[2] Ibrahim D, Zhu H, Yusof N, Isnaeni, Lim S-H. *Bacillus licheniformis* BT5.9 isolated from changar hot spring, malang, indonesia, as a potential producer of thermostable α-amylase. Trop Life Sci Res. 2013;24(1):71–84.

Table 1S. The separation of total lipids by HILIC, including the description of lipid classes, their abundance (percentage of total lipids) and retention times of individual lipid classes (tR).

tR (min)	*Alicyclobacillus acidoterrestris*	*Bacillus licheniformis*	*Geobacillus stearothermophilus*	Lipid
13.93	1.7 ± 0.4[a]	1.5 ± 0.4	1.2 ± 4.0	FFA
18.02	4.2 ± 0.9	21.0 ± 4.7	23.1 ± 4.6	PG
18.37	0.0 ± 0.0	**1.1 ± 0.3**	**1.4 ± 0.7**	**allo-Ile-PG[b]**
18.49	0.0 ± 0.0	**1.9 ± 0.5**	**3.2 ± 0.6**	**Ile-PG**
18.88	0.0 ± 0.0	**2.5 ± 0.7**	**3.9 ± 1.1**	**Leu-PG**
19.53	**2.5 ± 0.7**	**4.0 ± 1.3**	**1.4 ± 0.4**	**Ala-PG**
20.32	27.6 ± 4.8	18.8 ± 3.5	19.6 ± 3.3	CL
21.75	**8.9 ± 2.3**	**2.3 ± 1.0**	**2.1 ± 0.7**	**Ala-CL**
22.27	0.0 ± 0.0	0.6 ± 0.3	0.9 ± 0.3	PC
23.58	17.2 ± 2.5	11.6 ± 2.8	7.8 ± 1.7	PE
24.77	**13.3 ± 1.9**	**2.5 ± 0.8**	**2.8 ± 0.9**	**Lys-PG**
27.02	**1.5 ± 0.7**	**4.0 ± 1.1**	**4.2 ± 1.6**	**Glc-CL**
27.76	0.6 ± 0.3	0.6 ± 0.2	0.7 ± 0.3	PI
28.34	0.0 ± 0.0	**1.7 ± 0.4**	**2.5 ± 1.0**	**Leu-CL**
28.81	0.0 ± 0.0	**2.3 ± 0.3**	**3.5 ± 1.2**	**allo-Ile-CL**
29.12	0.0 ± 0.0	**2.7 ± 0.5**	**3.9 ± 1.4**	**Ile-CL**
31.03	**3.3 ± 0.8**	**3.6 ± 0.7**	**2.1 ± 0.8**	**Lys-CL**
32.04	**4.2 ± 1.3**	**0.3 ± 0.2**	**0.6 ± 0.2**	**allo-Thr-PG**
32.32	**5.3 ± 1.4**	0.0 ± 0.0	0.0 ± 0.0	**Thr-PG**
34.17	0.8 ± 0.3	0.7 ± 0.2	0.5 ± 0.2	PA
35.37	0.8 ± 0.3	0.4 ± 0.2	0.3 ± 0.1	PS
39.52	**2.2 ± 0.6**	0.0 ± 0.0	0.0 ± 0.0	**allo-Thr-CL**
39.90	**2.5 ± 0.5**	0.0 ± 0.0	0.0 ± 0.0	**Thr-CL**
44.56	3.4 ± 1.0	0.8 ± 0.3	1.6 ± 0.7	Lys-Glc-CL
45.27	0.0 ± 0.0	2.9 ± 1.2	2.1 ± 0.9	(Glc)2-CL
48.12	0.0 ± 0.0	6.7 ± 2.9	3.9 ± 1.4	(Glc)3-CL
53.29	0.0 ± 0.0	4.0 ± 0.8	4.4 ± 1.3	(Glc)4-CL
58.04	0.0 ± 0.0	1.5 ± 0.7	2.3 ± 0.8	(Glc)5-CL

[a] Data are presented as arithmetic means from three chromatographic analyses.

[b] Lipid classes marked in bold were separated by HILIC and further analyzed.

Table 2S. Results of the molecular composition analysis of three complex lipids (Ala-CL, Lys-CL, and Thr-CL) using MS^1.

Ala-CL			Lys-CL			Thr-CL		
$[M-H]^-$	Relative abundance (%)	Sum $FA_1+FA_2+FA_3+FA_4$	$[M-H]^-$	Relative abundance (%)	Sum $FA_1+FA_2+FA_3+FA_4$	$[M-H]^-$	Relative abundance (%)	Sum $FA_1+FA_2+FA_3+FA_4$
1340.88740	0.5	56	1367.93470	0.4	56	1340.88743	0.2	56
1354.90305	1.1	57	1381.95035	1.3	57	1354.90308	2.3	57
1368.91870	4.8	58	1395.96600	5.2	58	1368.91873	4.1	58
1382.93435	5.9	59	1409.98165	6.5	59	1382.93438	10.8	59
1396.95000	24.7	60	1423.99730	24.7	60	1396.95003	15.7	60
1410.96565	28.5	61	1438.01295	28.6	61	1410.96568	28.6	61
1424.98130	32.3	62	1452.02860	32.5	62	1424.98133	25.8	62
1438.99695	86.6	63	1466.04425	87.0	63	1438.99698	87.0	63
1453.01260	97.3	64	1480.05990	97.4	64	1453.01263	67.4	64
1467.02825	89.2	65	1494.07555	89.6	65	1467.02828	100.0	65
1481.04390	89.8	66	1508.09120	89.6	66	1481.04393	75.1	66
1495.05955	100.0	67	1522.10685	100.0	67	1495.05958	82.3	67
1509.07520	63.4	68	1536.12250	63.6	68	1509.07523	54.8	68
1523.09085	39.2	69	1550.13815	39.0	69	1523.09088	49.2	69
1537.10650	41.4	70	1564.15380	41.6	70	1537.10653	30.5	70
1551.12215	29.0	71	1578.16945	28.6	71	1551.12218	23.5	71
1565.13780	10.8	72	1592.18510	10.4	72	1565.13783	14.2	72
1579.15345	7.5	73	1606.20075	7.8	73	1579.15348	5.7	73
1593.16910	2.2	74	1620.21640	2.6	74	1593.16913	4.1	74
1607.18475	1.1	75	1634.23205	1.3	75	1607.18478	1.1	75
1621.20040	0.5	76	1648.24770	0.6	76	1621.20043	0.9	76

Table 3S. Commonly occurring product ions for allo-Ile-PG or Ile-PG or Leu-PG(15:0/17:0)

m/z	Ion Description	Abundance
130.0876	[allo-Ile-H]$^-$	17
152.9959	Glycerol-3-phosphate ion with loss of H_2O	5
169.0272	Glycerol-3-phosphate ion	11
209.0222	Glycerophosphoglycerol ion -2H_2O	6
227.0327	Glycerophosphoglycerol ion -H_2O	3
241.2174	sn-1 RCOO$^-$ ion	100
245.0433	Glycerophosphoglycerol ion	4
269.2487	sn-2 RCOO$^-$ ion	45
377.2100	Neutral loss of sn-2 RCOOH group and glycerol from [M-H]$^-$	13
395.2205	Loss of sn-2 acyl chain as ketene (RCH=C=O) and glycerol from [M-H]$^-$	8
405.2414	Neutral loss of sn-1 RCOOH group and glycerol from [M-H]$^-$	7
423.2519	Loss of sn-1 acyl chain as ketene (RCH=C=O) and glycerol from [M-H]$^-$	3
451.2468	Neutral loss of sn-2 RCOOH group from [M-H]$^-$	7
469.2574	Loss of sn-2 acyl chain as ketene (RCH=C=O) from [M-H]$^-$	9
479.2781	Neutral loss of sn-1 RCOOH group from [M-H]$^-$	6
497.2887	Loss of sn-1 acyl chain as ketene (RCH=C=O) from [M-H]$^-$	5
647.4659	Loss of glycerol and AA from precursor ion	5
703.4921	Loss of AA from precursor ion	3
834.5868	Precursor ion [M-H]$^-$	7

Table 4S. Retention characteristics of amino acid standards.

Amino acid (AA)	tR of L-AA (min)	tR of D-AA (min)	α
allo-Thr	22.45	23.40	1.042
Thr	22.63	24.93	1.102
Ala	24.44	26.96	1.103
Ile	34.93	38.93	1.115
allo-Ile	35.25	39.14	1.110
Leu	35.75	39.36	1.101
Lys	41.78	42.79	1.024

tR - retention time, see Experimental, α - separation factors of the (S)-NIFE derivatives.

Table 5S. Examples of relevant enzymes listed in GenBank that biosynthesize amino acyl lipids in three cultured bacteria.

Enzyme	Bacterium	Gen Bank
isoleucine-tRNA ligase	*Geobacillus stearothermophilus*	KMY58884.1
isoleucyl-tRNA synthetase	*Bacillus licheniformis*	KYD01040.1
threonine-tRNA ligase	*Alicyclobacillus acidoterrestris*	UNO48242.1

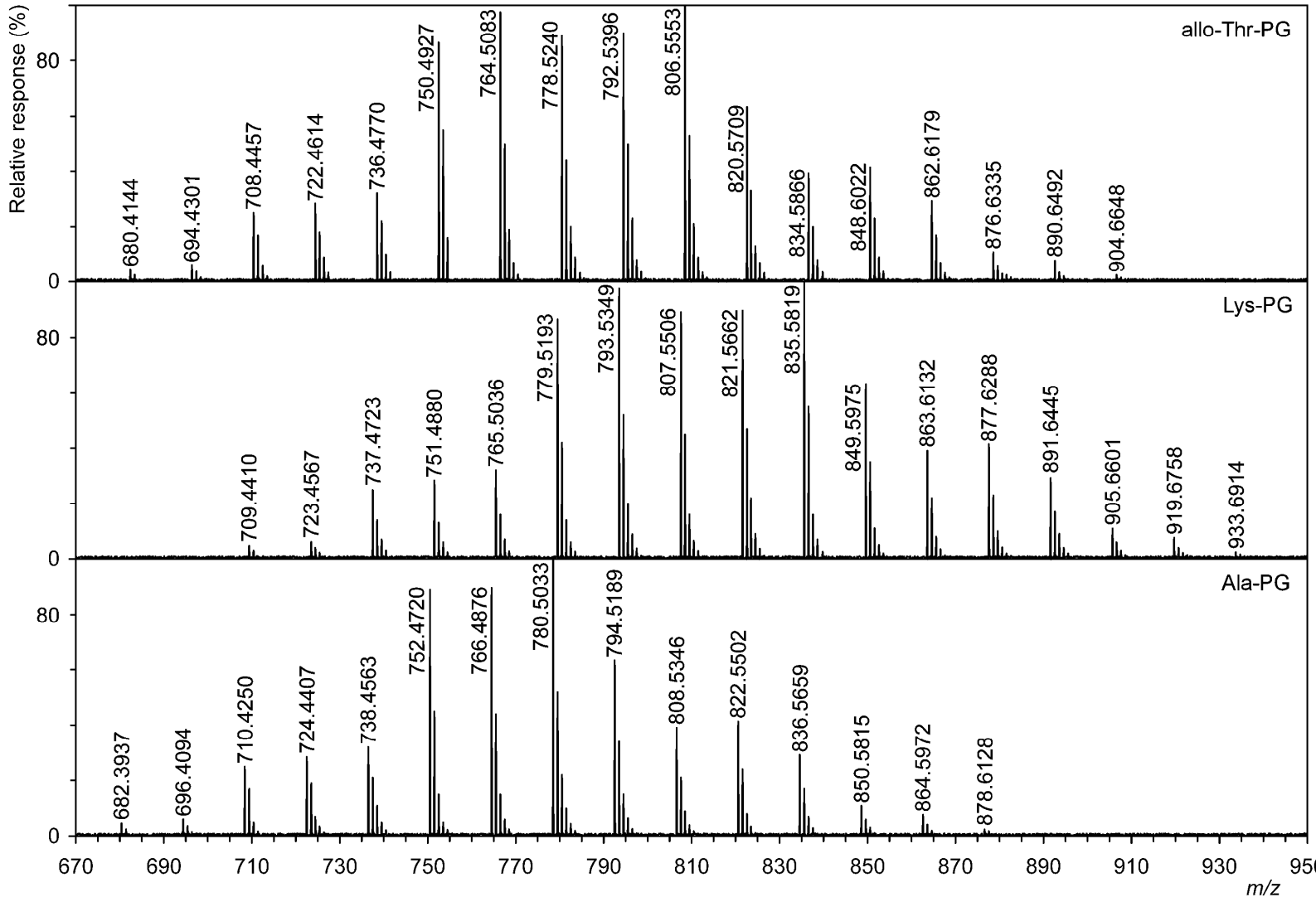


Fig. 1S. Shotgun analysis of a mixture of AA-PGs from thermophilic bacteria isolated from hot springs. Numbers correspond to the accurate mass values.

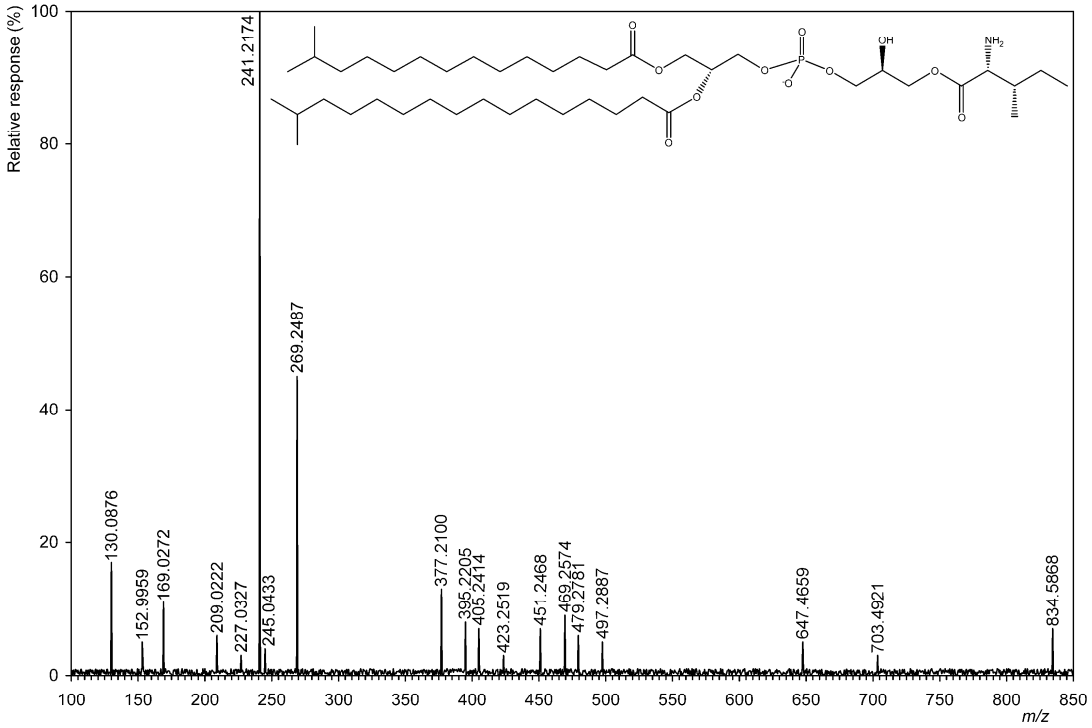


Fig. 2S. Tandem mass spectrum of aminoacylated PG (allo-Ile-PG or Ile-PG or Leu-PG). Description of ions (their structures) and abundances including *m/z* values are given in Table 3S.

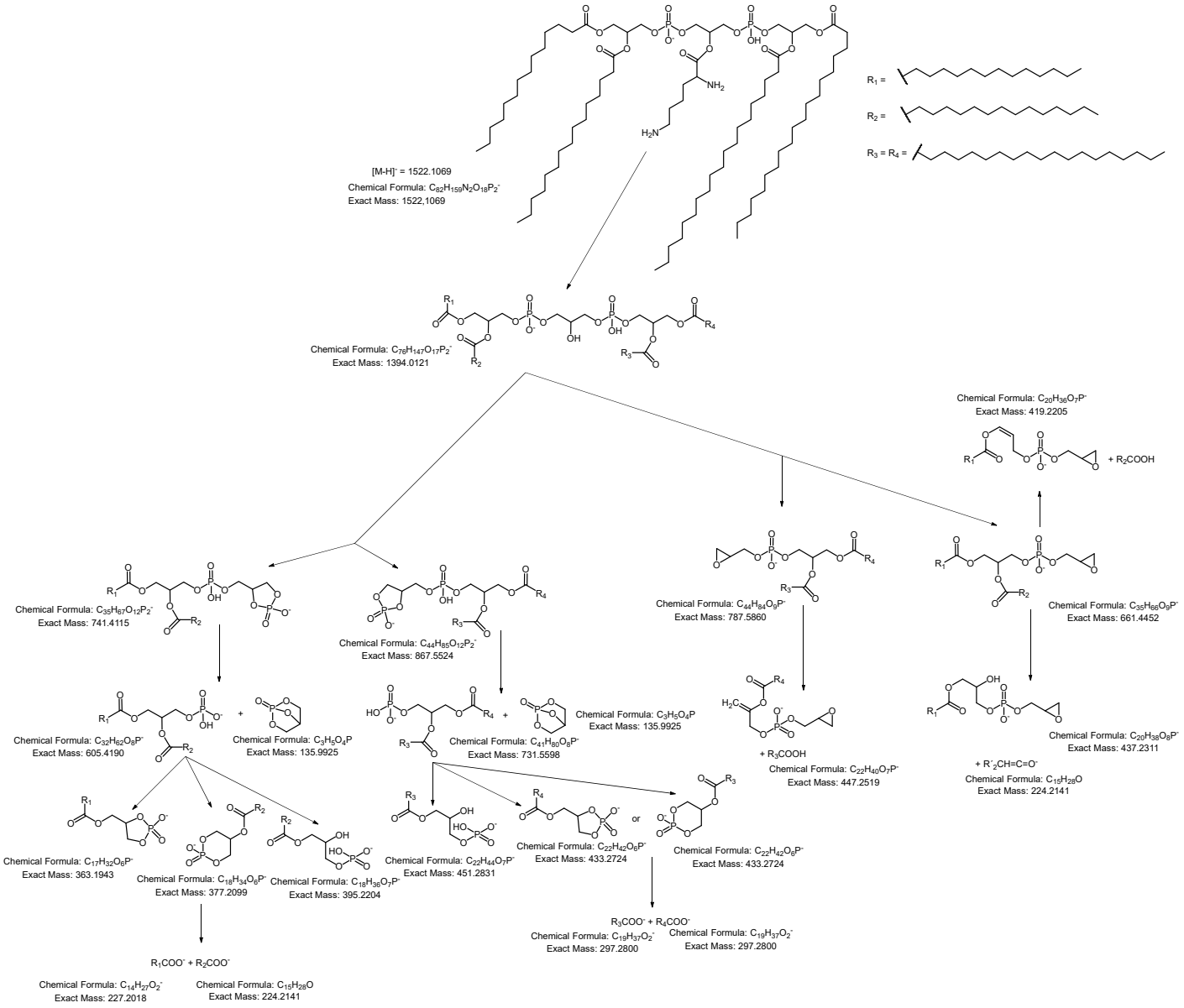
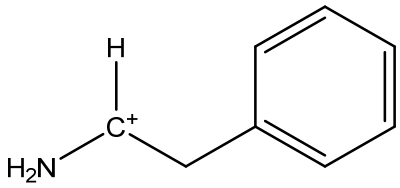


Fig. 3S. Probable major fragmentation pathways of Lys-14:0/15:0-19:0/19:0–CL.



Chemical Formula: $C_8H_{10}N^+$
Exact Mass: 120.0809

Fig. 4S. A probable structure of fragment ion at *m/z* 120.080, see also the already published paper (DOI: 10.1038/srep46289).