Supplements

Analysis of cardiolipins by RP-HPLC/MS, phospholipase C and chiral chromatography

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2. Experimental Methods

2.2. Analyzed samples **-** Cultivation of the green alga *Chlamydomonas reinhardtii*

The green alga *Chlamydomonas reinhardtii* (wild type CC-125) was obtained from the Chlamydomonas Resource Center at the University of Minnesota (St. Paul, MN, USA). It was cultivated on high salt (HS) nutrient medium as described by Sueoka [1]. Microelements (1 mL per 1 L of medium) were added as described by Zachleder and Šetlík [2]. A flat glass photobioreactor containing 2500 mL of liquid HS medium was inoculated directly from stock plates. The photobioreactor was illuminated by fluorescent tubes (OSRAM DULUX L55W/950 Daylight, Milano, Italy); the incident light intensity was 750 μmol m^{-2} s^{-1} of photosynthetically active radiation. The cultures were grown for 3 days at 30°C, aerated with 2% (v/v) CO_2 in air. Then the biomass was harvested by centrifugation, frozen at -80°C and freeze-dried.

2.4. Preparation and analysis of fatty acid methyl esters

A ~1 mg of total lipids was dissolved in 1 mL diethyl ether and 3 mL methyl acetate. 25 μL of 1 M sodium methoxide in methanol were added. The sample was incubated for 50 min at room temperature. Saturated oxalic acid solution (0.5 mL) was added, with brief agitation, to neutralize the solution. The solvent was removed under nitrogen, and an appropriate volume of hexane was added to bring the sample to the concentration required for analysis [21].

Splitless injection was at 100 °C, and a fused silica capillary column (Supelcowax 10; 60 m x 0.25 mm i.d., 0.25 mm film thickness; Supelco, Prague) was used. The temperature program was as follows: 100 °C for 1 min, subsequently increasing at 20 °C/min to 180 °C and at 2 °C/min to 280 °C, which was maintained for 1 min. The carrier gas was helium at a linear velocity of 60 cm/s. All spectra were scanned within the range of *m/z* 50–500. FAMEs were identified according to their mass spectra [25, 26] and using a mixture of chemical standards obtained from Merck.

2.10 Lipid nomenclature

Lipid nomenclature was applied according to Liebisch et al. [31], see Supplementary Information. The lipid's total FA composition is described with x for total carbon number and

y for total number of double bonds within a lipid class (i.e. 72:4-CL). The individual FA moiety is specified via a slash for a specific position (i.e. x1:y1/x2:y2/x3:y3/x4:y4-CL; 22:0/24:0/24:1/22:1-CL) and via an underscore for unknown positions (i.e. x1:y1_x2:y2_x3:y3_x4:y4-CL; 22:0_24:0_24:1_22:1-CL) on the glycerol backbone.

2.11. Statistics

The statistical analysis was performed using the IBM SPSS Statistics (Statistical Package for the Social Sciences; IBM Corp, 2013) Statistics software version 26 (IBM® Corporation, Armonk, NY, USA), to explore the metabolic relationships between different sources of carbons. Three replicates (n = 3) of each analysis were used. Data were presented as means ± SD.

The control of the LC-MS instrumentation and the first processing of data were performed by the Xcalibur software 2.2 SP1.48 (Thermo Scientific). The ChemDraw 20 software (CambridgeSoft Corporation, Cambridge, MA, USA) was employed to draw chemical structures.

References

[1] N. Sueoka, Mitotic replication of deoxyribonucleic acid in *Chlamydomonas reinhardtii*, Proc. Natl. Acad. Sci. USA 46 (1960) 83–91.

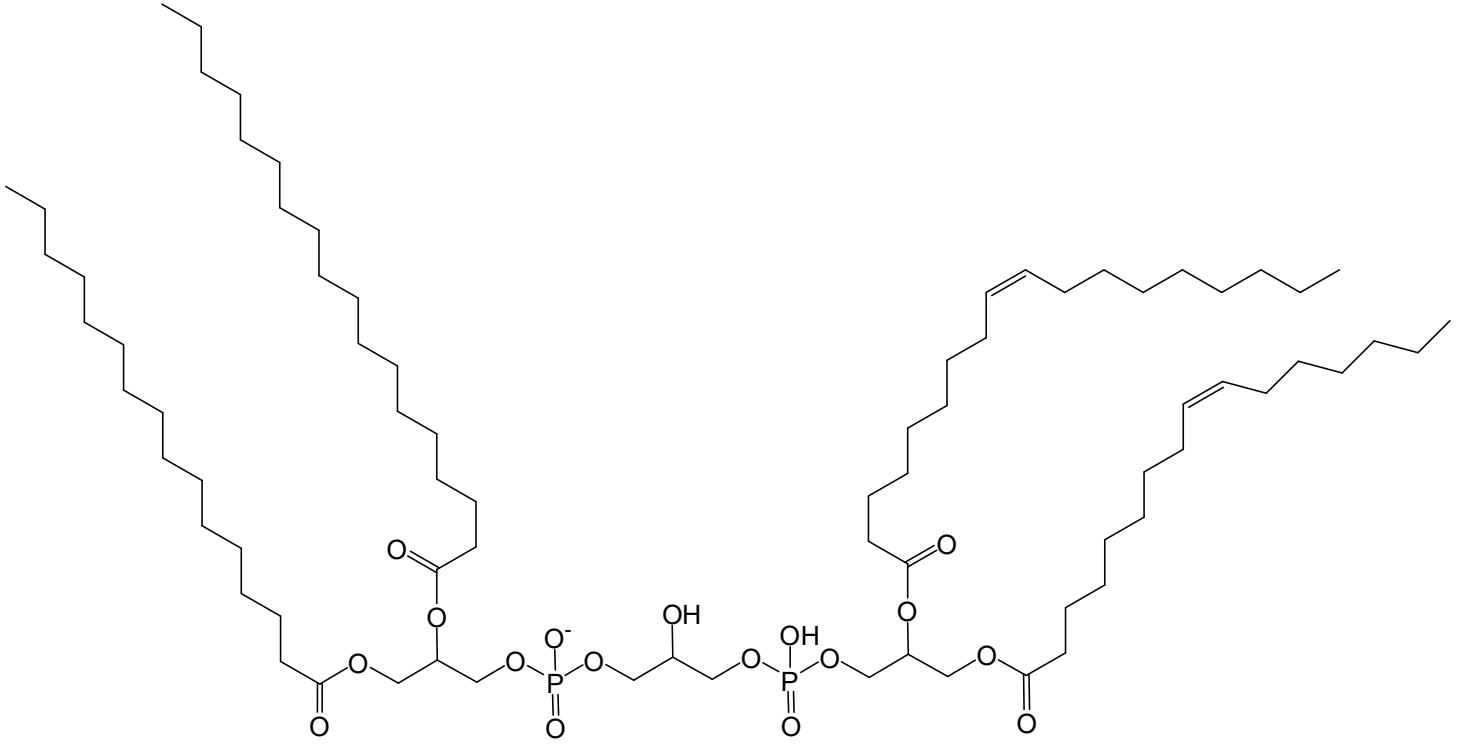
[2] V. Zachleder, I. Šetlík, Effect of irradiance on the course of RNA synthesis in the cell cycle of *Scenedesmus quadricauda*, Biol. Plant. 24 (1982**)** 341–353.

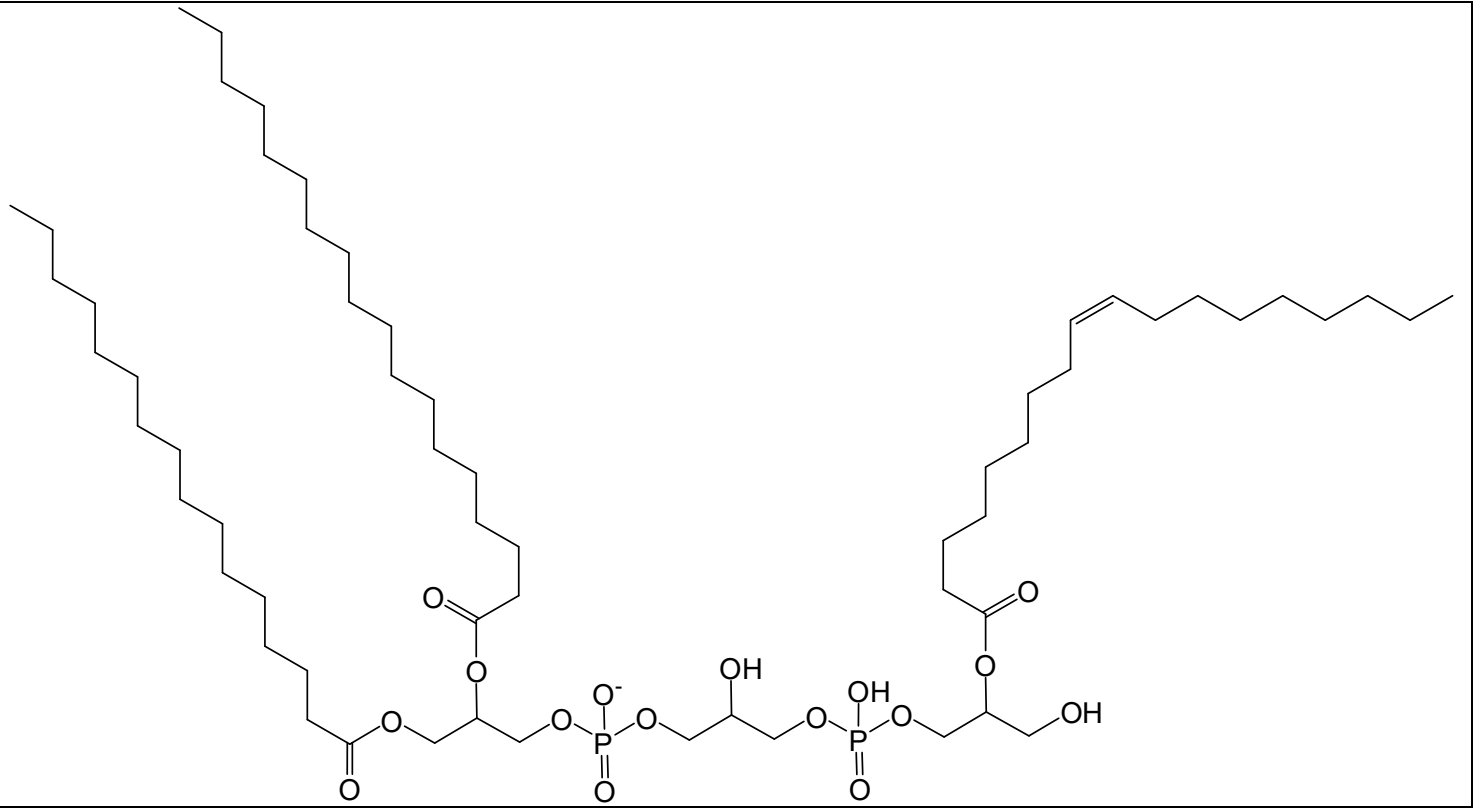
Table 1S. Fatty acid content from four sources (Gram positive bacterium *Kocuria rhizophila* CCM 552, green alga *Chlamydomonas reinhardtii* CC-125, bottom brewer's yeast *Saccharomyces pastorianus,* spinach and bovine heart).

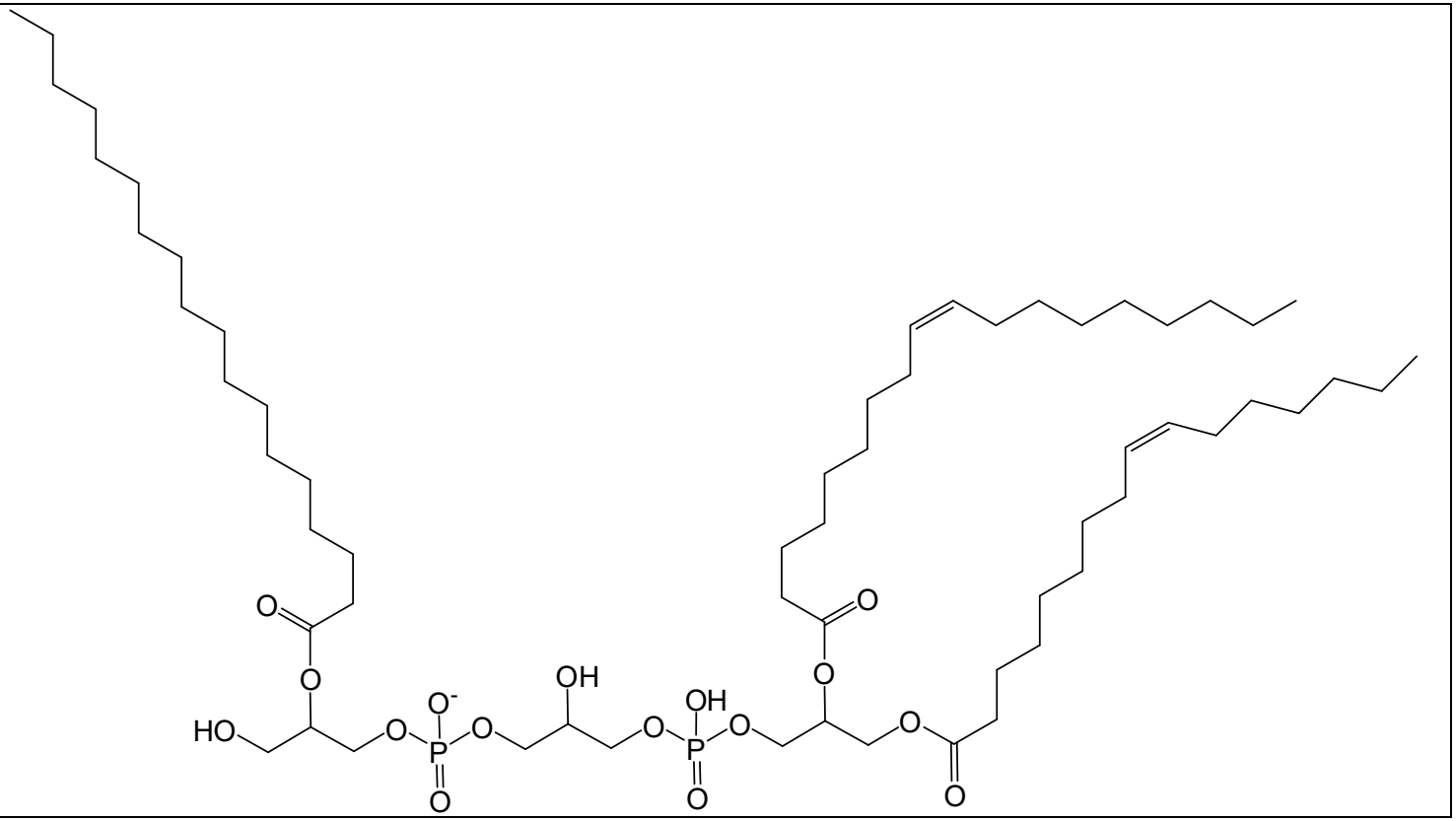
FA	bacterium	yeast	alga	spinach	heart
14:0	9.2±0.7[a]	2.5±0.5	1.1±0.2	-	-
15:0	40.4±1.5	-	-	-	-
16:0	30.4±2.2	10.7±1.8	12.1±1.4	10.3±0.5	1.7±0.6
16:1	5.9±1.0	43.4±2.1	18.4±1.0	2.6±0.4	3.4±0.4
16:2	-	-	5.4±0.5	-	-
16:3	-	-	2.7±0.2	-	-
16:4	-	-	4.8±0.3	-	-
17:0	9.5±0.9	-	-	-	0.8±0.1
17:1	1.6±0.4	-	-	-	1.3±0.5
18:0	1.8±0.2	4.9±0.9	13.2±1.6	5.2±0.6	1.3±0.4
18:1	1.2±0.3	31.0±1.0	16.5±1.8	9.5±0.7	7.1±0.6
18:2	-	-	10.4±0.6	32.9±3.5	75.7±2.7
18:3	-	-	15.4±1.1	39.5±2.3	4.1±1.2
20:0	-	1.7±0.1	-	-	-
20:1	-	1.4±0.3	-	-	-
20:4	-	-	-	-	2.7±0.3
20:5	-	-	-	-	1.9±0.4
22:0	-	0.8±0.2	-	-	-
22:1	-	1.1±0.2	-	-	-
24:0	-	0.8±0.3	-	-	-
24:1	-	0.9±0.5	-	-	-
26:0	-	0.8±0.3	-	-	-

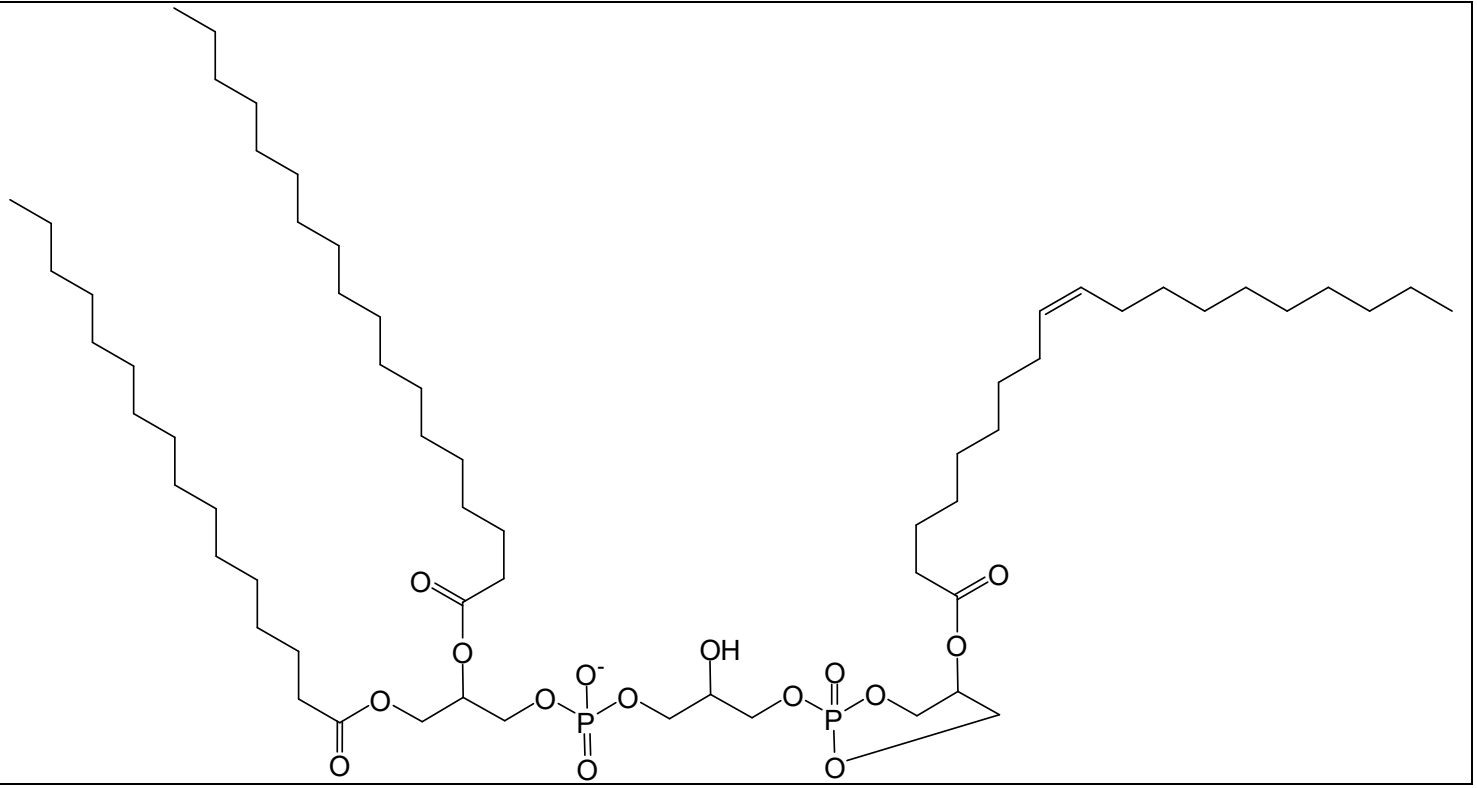
[a] Mean ± S.D. from three measurements, i.e. relative %.

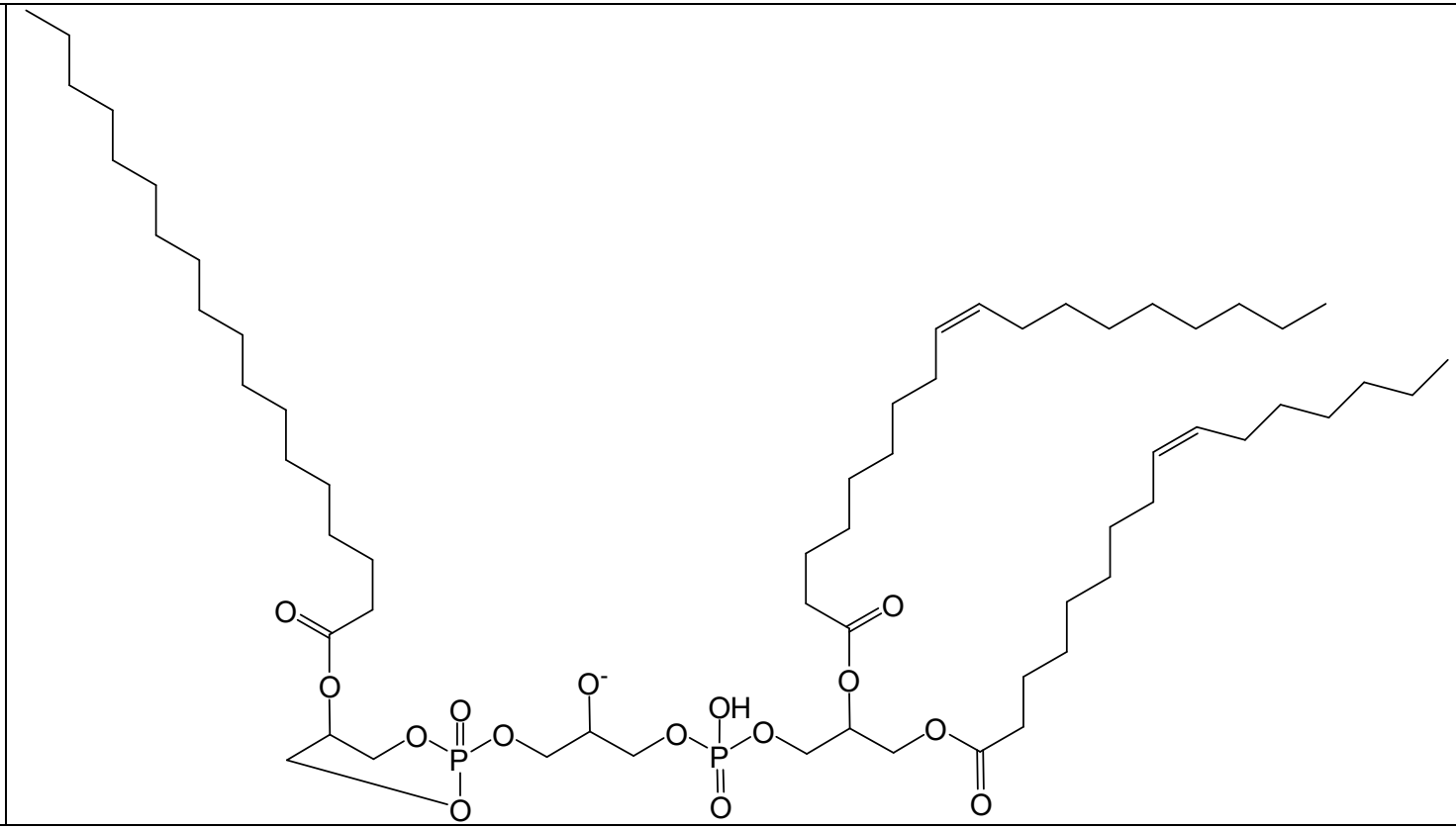
Table 2S. Tandem MS of 16:1_16_0_18:1_18:0-CL.

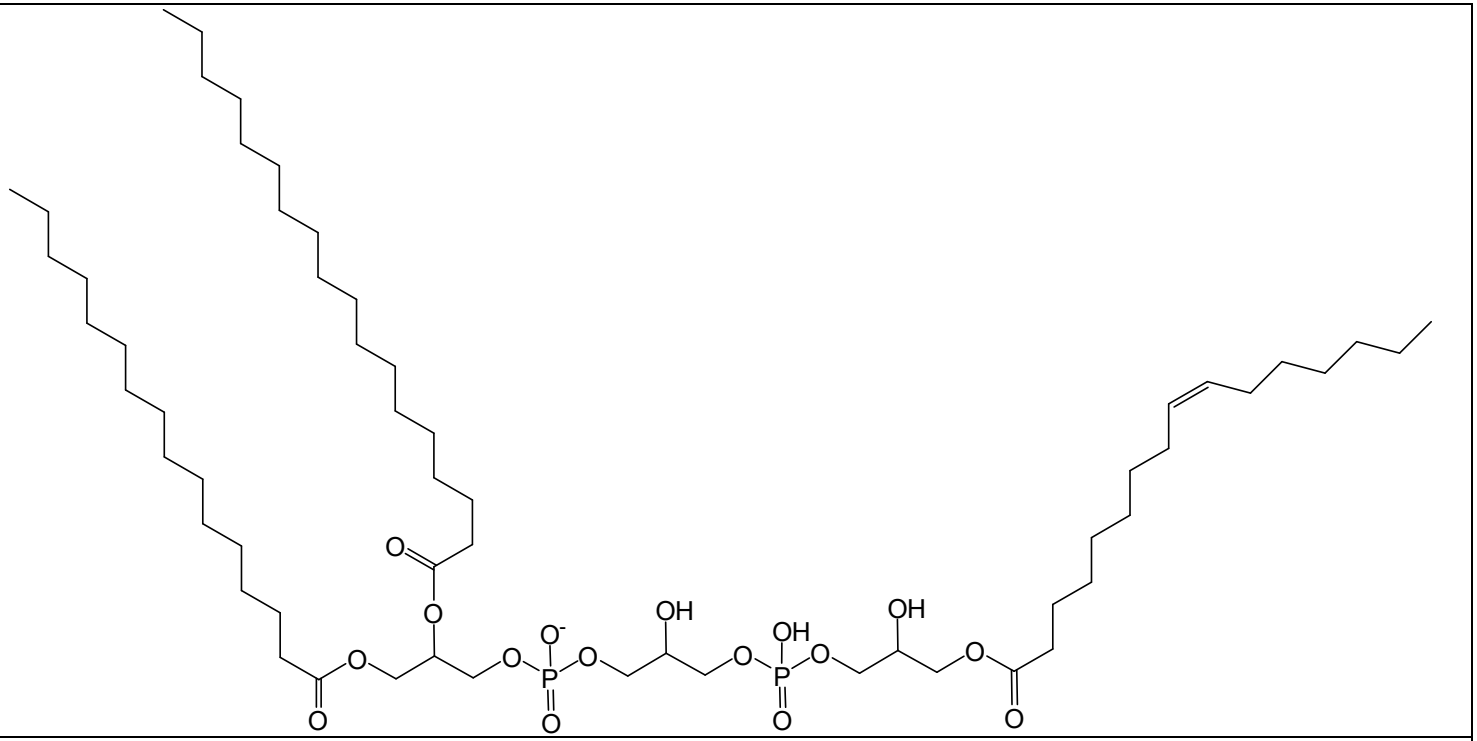
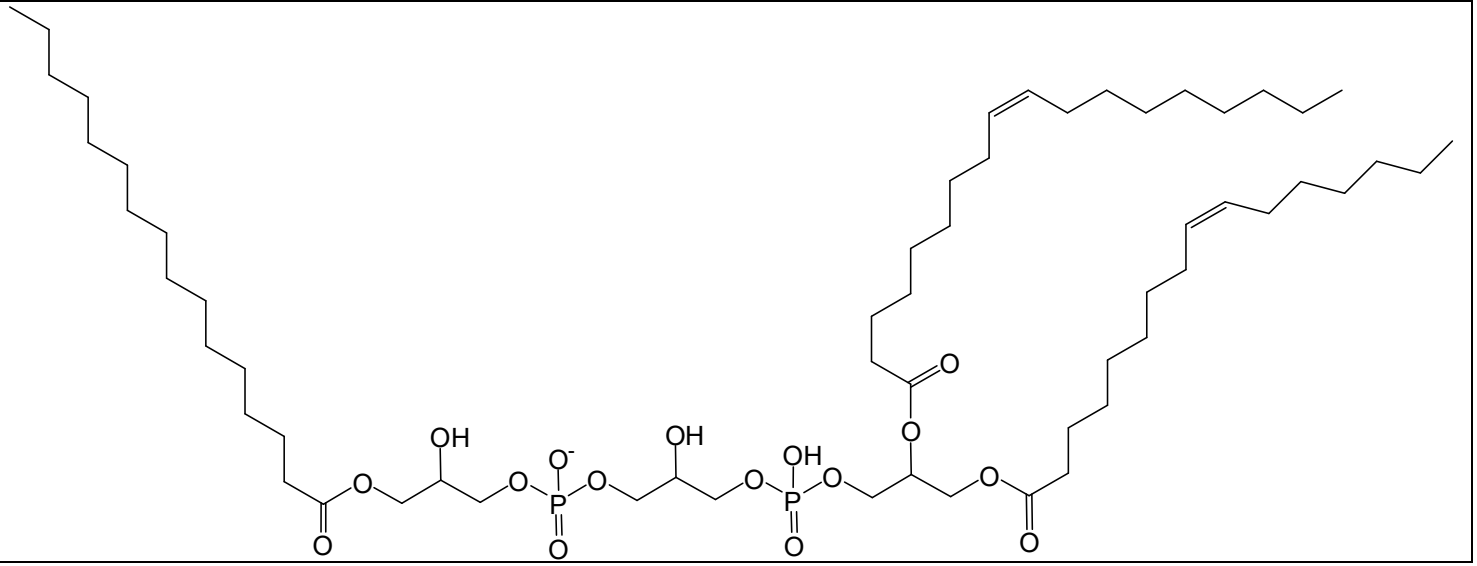
Exact Mass	Chemical Formula	Structure	
1403.9962	$C_{77}H_{145}O_{17}P_2^-$	$[M-H]^-$	

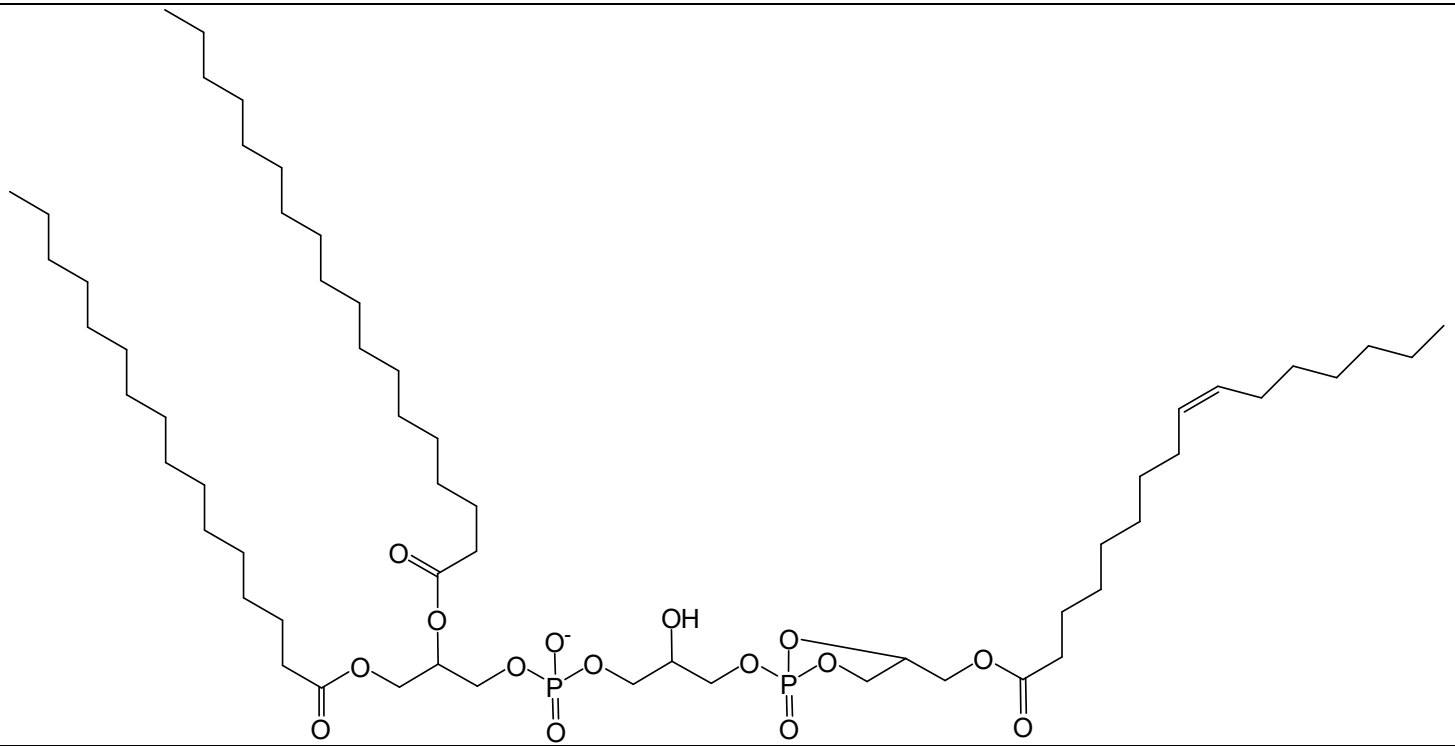
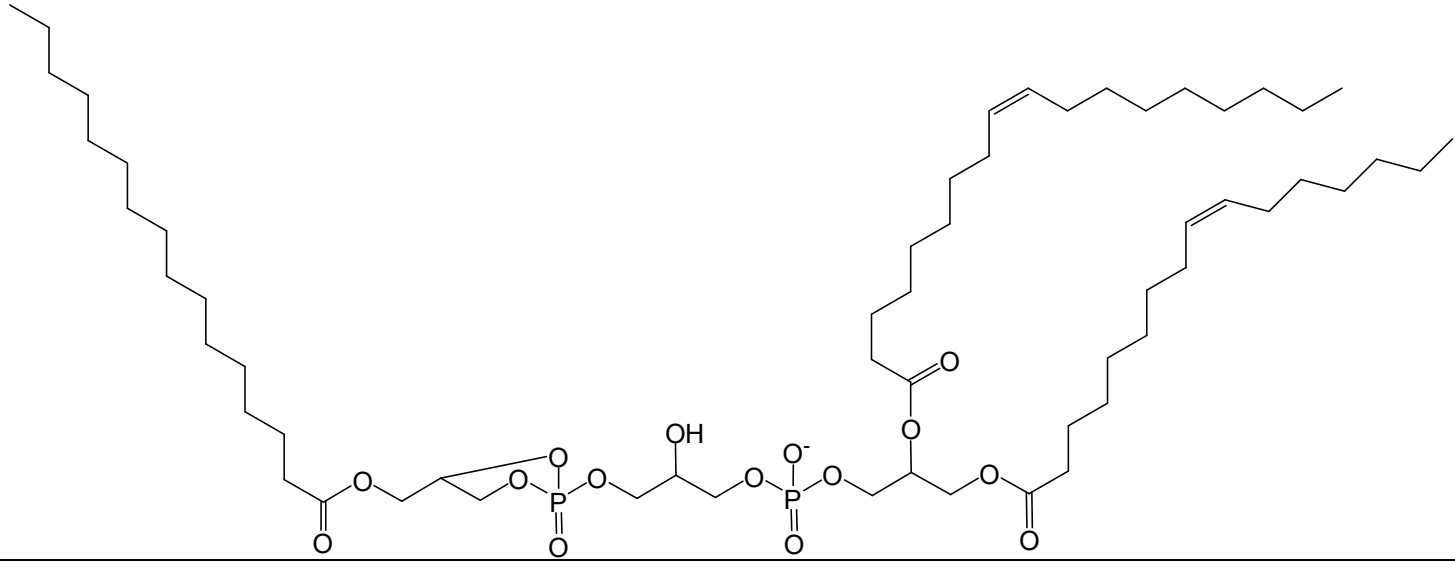
1167.7822	$C_{61}H_{117}O_{16}P_2^-$		

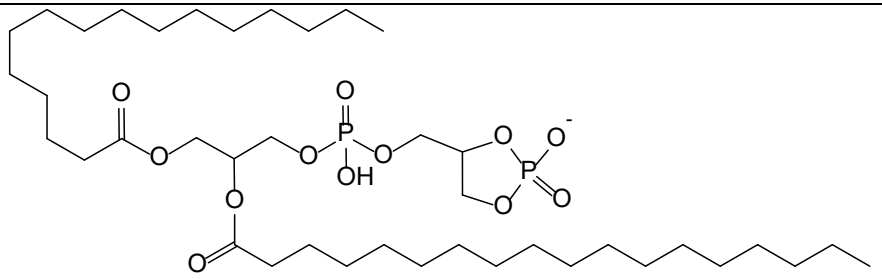
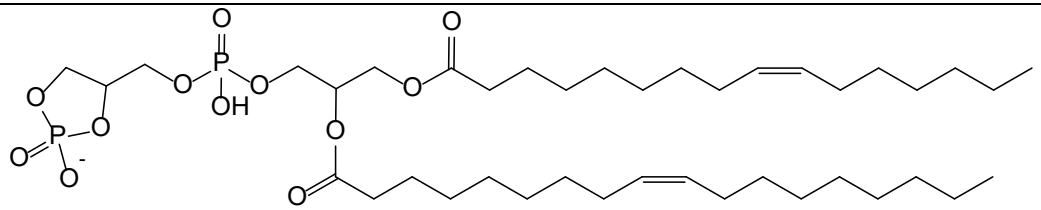
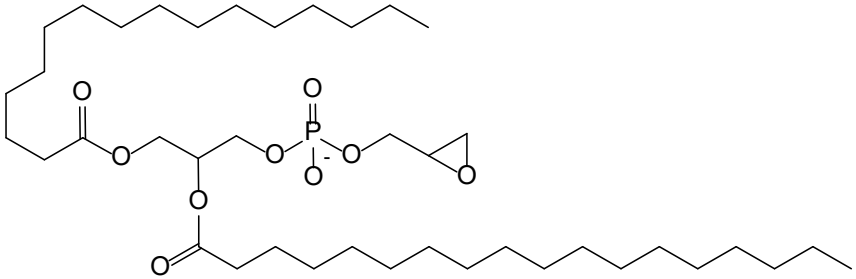
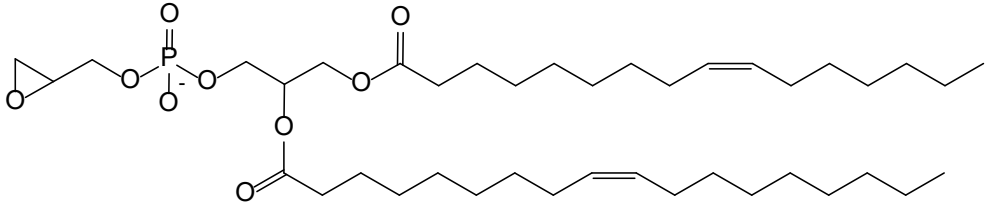
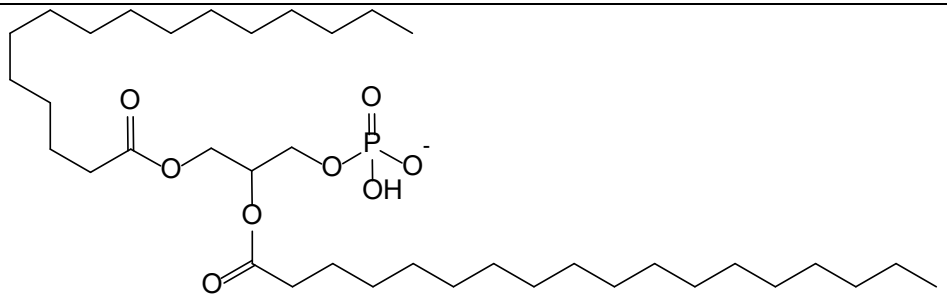
1165.7666	$C_{61}H_{115}O_{16}P_2^-$		

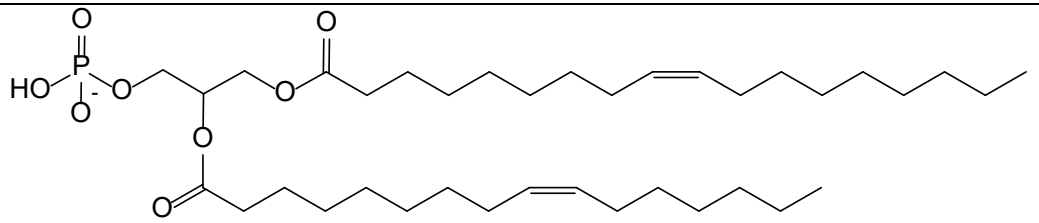
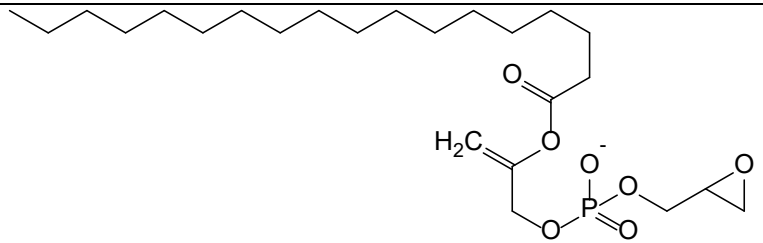
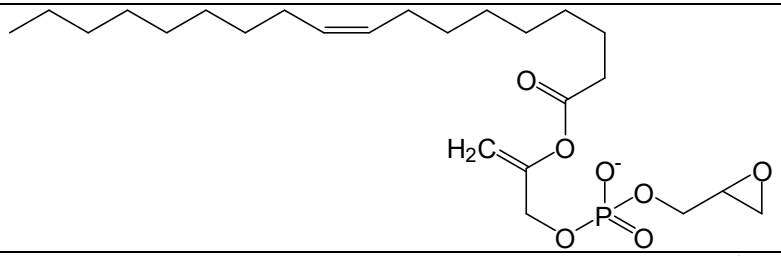
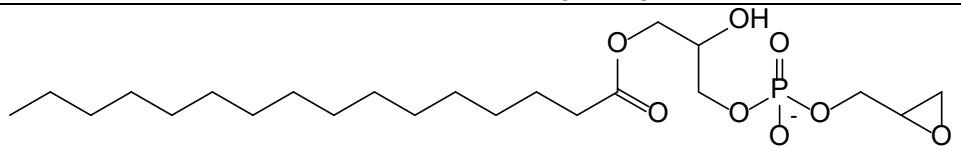
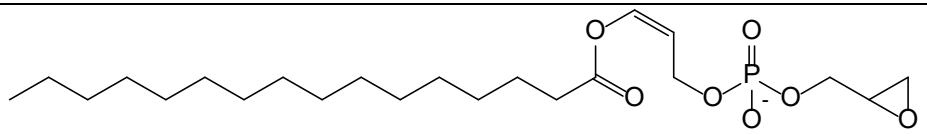
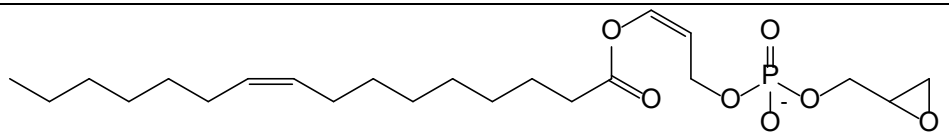
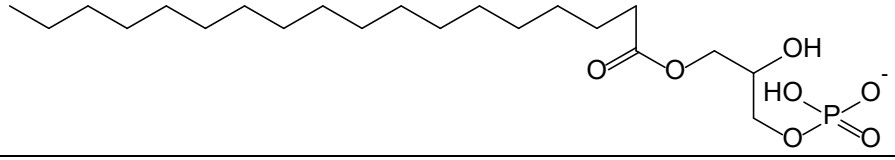
1149.7717	$C_{61}H_{115}O_{15}P_2^-$		

1147.7560	$C_{61}H_{113}O_{15}P_2^-$		

1139.7509	$C_{59}H_{113}O_{16}P_2^-$		
1137.7353	$C_{59}H_{111}O_{16}P_2^-$		

1121.7404	$C_{59}H_{111}O_{15}P_2^-$		
1119.7247	$C_{59}H_{109}O_{15}P_2^-$		

811.4896	$C_{40}H_{77}O_{12}P_2^-$	a+136	
807.4583	$C_{40}H_{73}O_{12}P_2^-$	b+136	
731.5232	$C_{40}H_{76}O_9P^-$	a+56	
727.4919	$C_{38}H_{68}O_9P^-$	b+56	
675.4970	$C_{37}H_{72}O_8P^-$	a	

671.4657	$C_{37}H_{68}O_8P^-$	b	
475.2830	$C_{24}H_{44}O_7P^-$		
473.2674	$C_{24}H_{42}O_7P^-$		
465.2623	$C_{22}H_{42}O_8P^-$		
447.2517	$C_{22}H_{40}O_7P^-$		
445.2361	$C_{22}H_{38}O_7P^-$		
437.2674	$C_{21}H_{42}O_7P^-$		

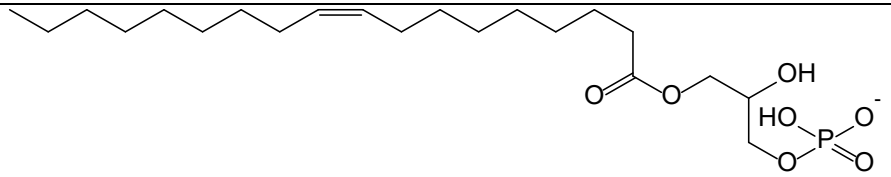
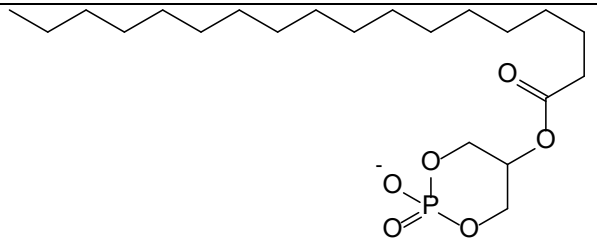
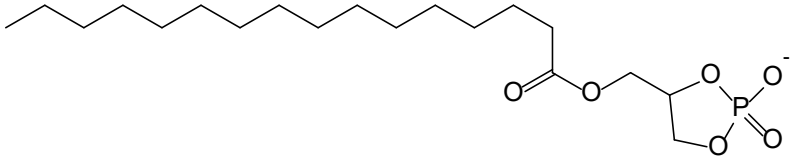
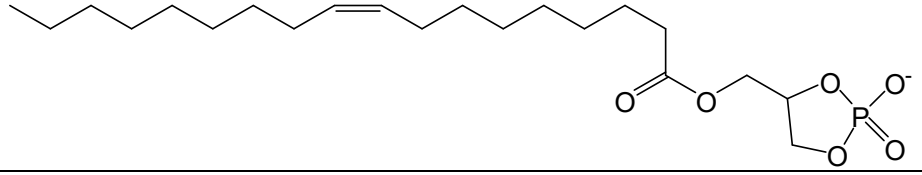
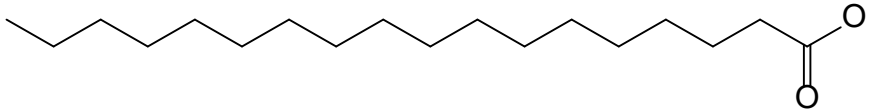
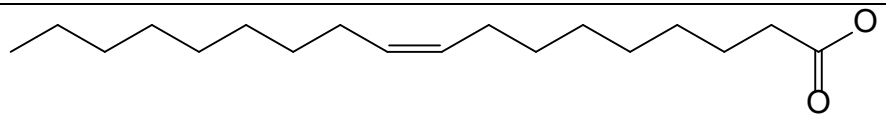
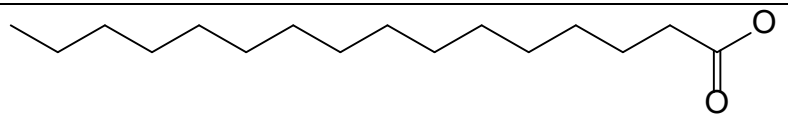
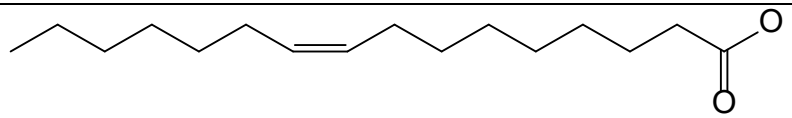
435.2517	$C_{21}H_{40}O_7P^-$		
419.2568	$C_{21}H_{40}O_6P^-$		
391.2255	$C_{19}H_{36}O_6P^-$		
389.2098	$C_{19}H_{34}O_6P^-$		
283.2643	$C_{18}H_{35}O_2^-$	S	
281.2486	$C_{18}H_{33}O_2^-$	O	
255.2330	$C_{16}H_{31}O_2^-$	P	
253.2173	$C_{16}H_{29}O_2^-$	Po	

Table 3Sa. Commonly occurring product ions for 18:2/16:0/2:0-TAG.

m/z	**abundance**	**Ion Description**
652.5511	33	Precursor ion $[M+NH_4]^+$
635.5245	5	Precursor ion $[M+H]^+$
575.5034	100	Neutral loss of sn-3 RCOOH + NH_3 from $[M+NH_4]^+$
379.2843	36	Neutral loss of sn-2 RCOOH + NH_3 from $[M+NH_4]^+$
355.2843	75	Neutral loss of sn-1 RCOOH + NH_3 from $[M+NH_4]^+$
337.2737	18	sn-1 acyl chain ($[RC=O+74]^+$)
319.2631	5	sn-1 acyl chain ($[RC=O+74]^+$ with loss of H_2O)
313.2737	15	sn-2 acyl chain ($[RC=O+74]^+$)
295.2631	6	sn-2 acyl chain ([RC=O +74]+ with loss of H_2O)
263.2369	16	sn-1 acyl chain ($[RC=O]^+$)
245.2264	5	sn-1 acyl chain ($[RC=O]^+$ with loss of H_2O)
239.2369	14	sn-2 acyl chain ($[RC=O]^+$)
221.2264	4	sn-2 acyl chain ($[RC=O]^+$ with loss of H_2O)

Table 3Sb. Commonly occurring product ions for 16:1/18:1/2:0-TAG.

m/z	abundance	Ion Description
652.5511	35	Precursor ion $[M+NH_4]^+$
635.5245	7	Precursor ion $[M+H]^+$
575.5034	100	Neutral loss of sn-3 RCOOH + NH_3 from $[M+NH_4]^+$
381.2999	81	Neutral loss of sn-1 RCOOH + NH_3 from $[M+NH_4]^+$
353.2686	33	Neutral loss of sn-2 RCOOH + NH_3 from $[M+NH_4]^+$
339.2894	20	sn-2 acyl chain ($[RC=O+74]^+$)
321.2788	9	sn-2 acyl chain ($[RC=O+74]^+$ with loss of H_2O)
311.2581	14	sn-1 acyl chain ($[RC=O+74]^+$)
293.2475	5	sn-1 acyl chain ($[RC=O+74]^+$ with loss of H_2O)
265.2526	18	sn-2 acyl chain ($[RC=O]^+$)
247.2420	7	sn-2 acyl chain ($[RC=O]^+$ with loss of H_2O)
237.2213	16	sn-1 acyl chain ($[RC=O]^+$)
219.2107	6	sn-1 acyl chain ($[RC=O]^+$ with loss of H_2O)

Table 3Sc. Commonly occurring product ions for 18:1/16:1/2:0-TAG.

m/z	abundance	Ion Description
652.5511	37	Precursor ion $[M+NH_4]^+$
635.5245	6	Precursor ion $[M+H]^+$
575.5034	100	Neutral loss of sn-3 RCOOH + NH_3 from $[M+NH_4]^+$
381.2999	33	Neutral loss of sn-2 RCOOH + NH_3 from $[M+NH_4]^+$
353.2686	72	Neutral loss of sn-1 RCOOH + NH_3 from $[M+NH_4]^+$
339.2894	20	sn-1 acyl chain ($[RC=O+74]^+$)
321.2788	6	sn-1 acyl chain ($[RC=O+74]^+$ with loss of H_2O)
311.2581	12	sn-2 acyl chain ($[RC=O+74]^+$)
293.2475	5	sn-2 acyl chain ($[RC=O+74]^+$ with loss of H_2O)
265.2526	18	sn-1 acyl chain ($[RC=O]^+$)
247.2420	6	sn-1 acyl chain ($[RC=O]^+$ with loss of H_2O)
237.2213	13	sn-2 acyl chain ($[RC=O]^+$)
219.2107	3	sn-2 acyl chain ($[RC=O]^+$ with loss of H_2O)

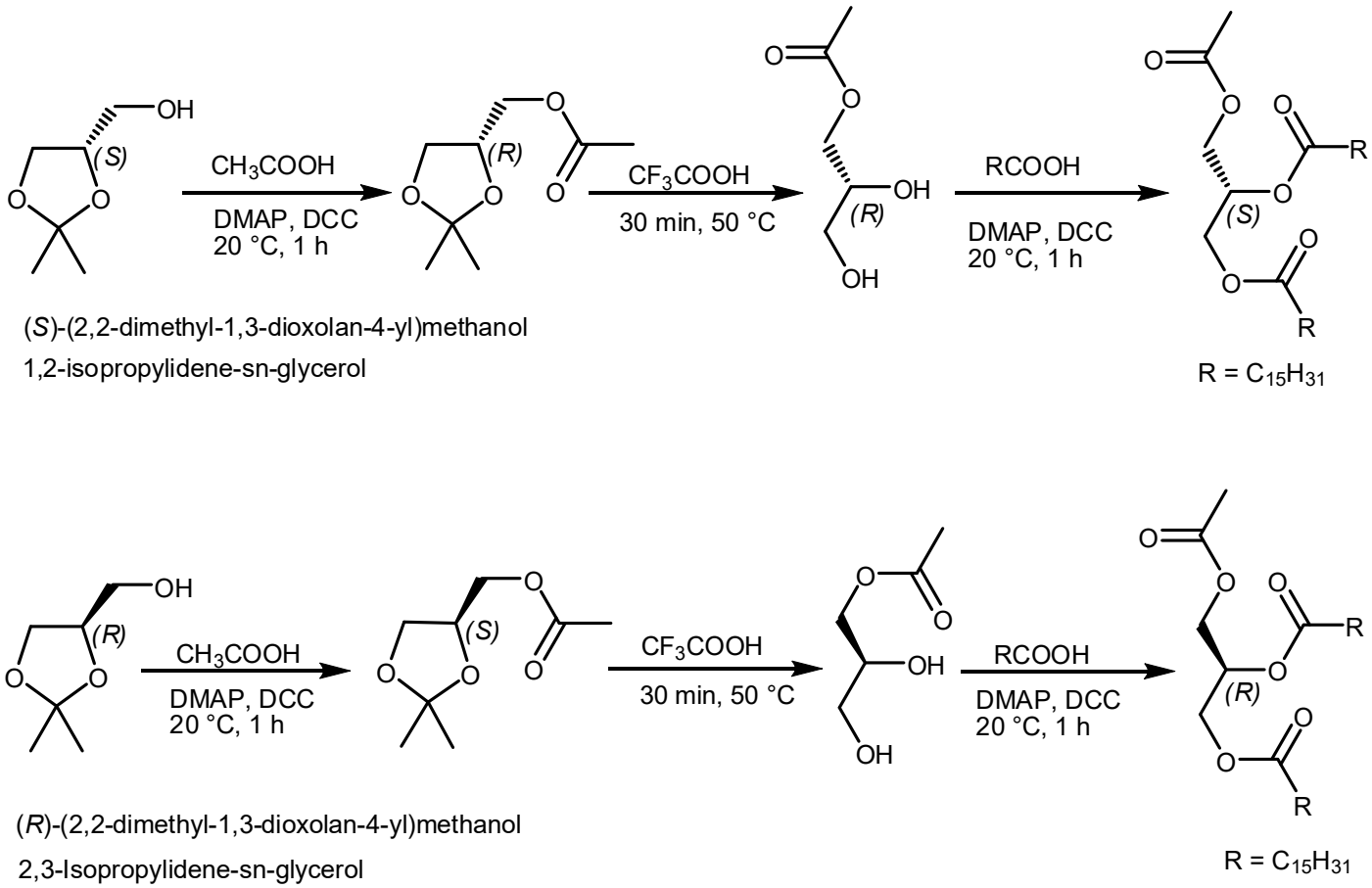


Fig. 1S. Synthesis of *R* and *S* enantiomers of dipalmitoyl-acetyl-glycerol.

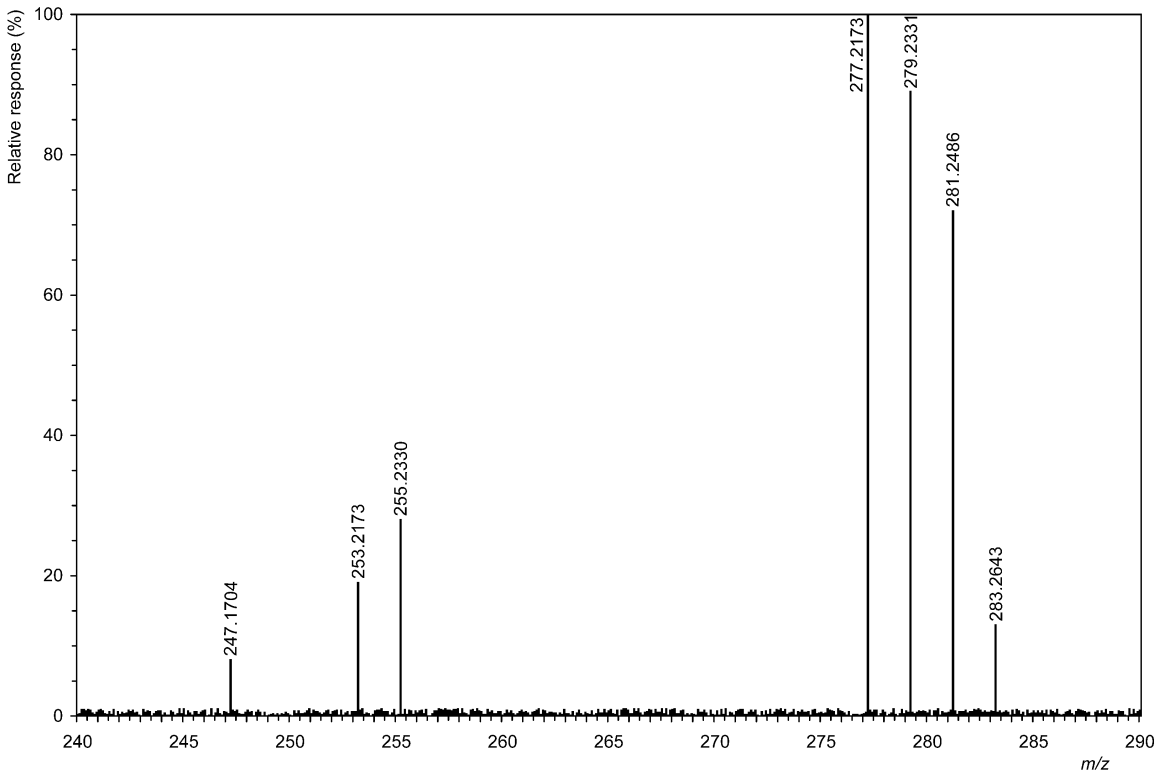


Fig. 2S. Tandem MS of a mixture of CLs at *m/z* 1395.9336 ($[M-H]^-$) where a total of seven $RCOO^-$ type ions could be identified (ions of the type $RCOO^-$ of appropriate fatty acids - 16:4 (247.1704 Da), 16:1 (253.2173 Da), 16:0 (255.2330 Da), 18:3 (277.2173 Da), 18:2 (279.2331), 18:1 (281.2486 Da), and 18:0 (283.2643 Da)).

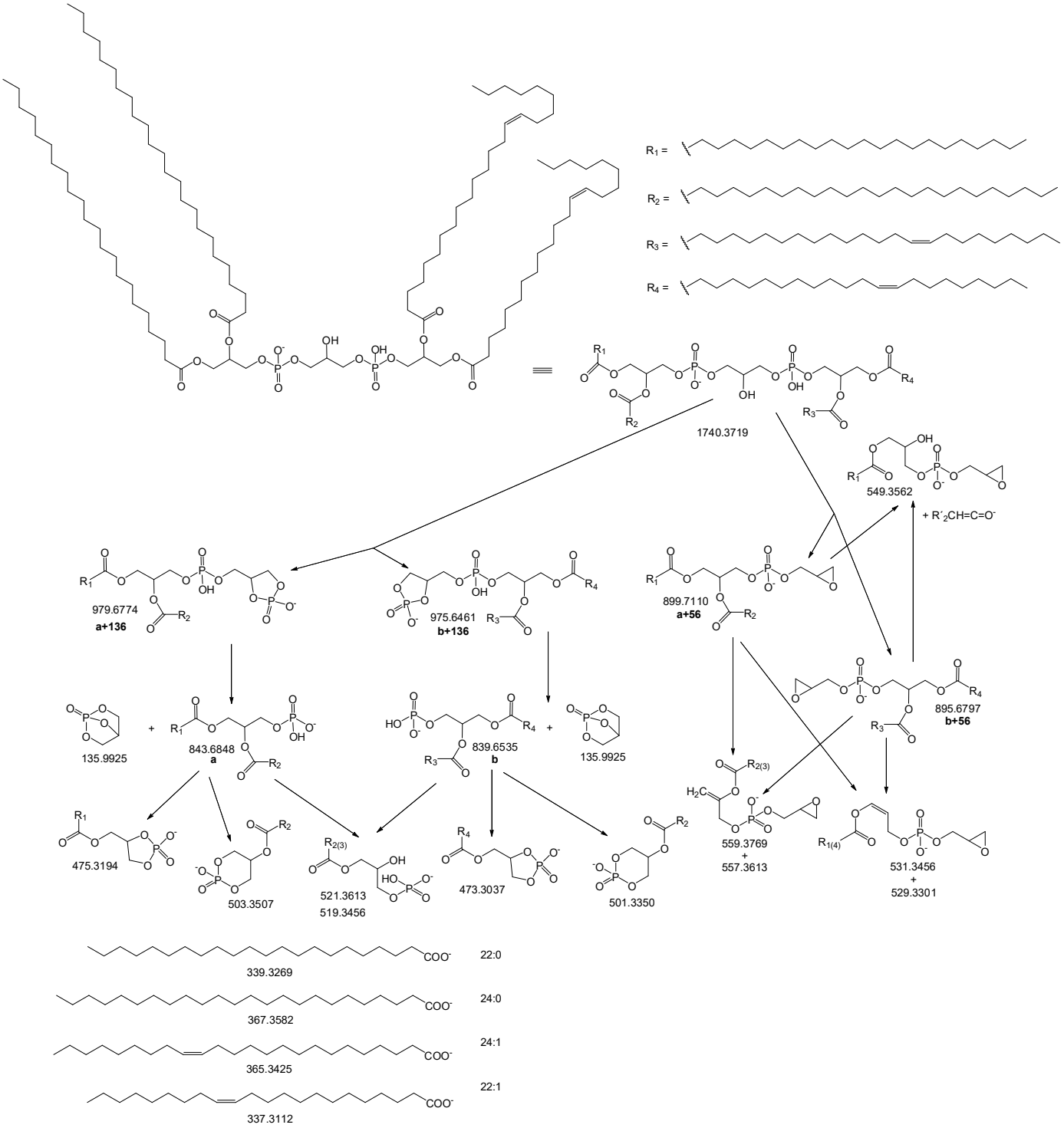


Fig. 3S. The major fragmentation pathways proposed for formation of ions from the $[M–H]^-$ ion of cardiolipin (22:0_24:0_24:1_22:1-CL).

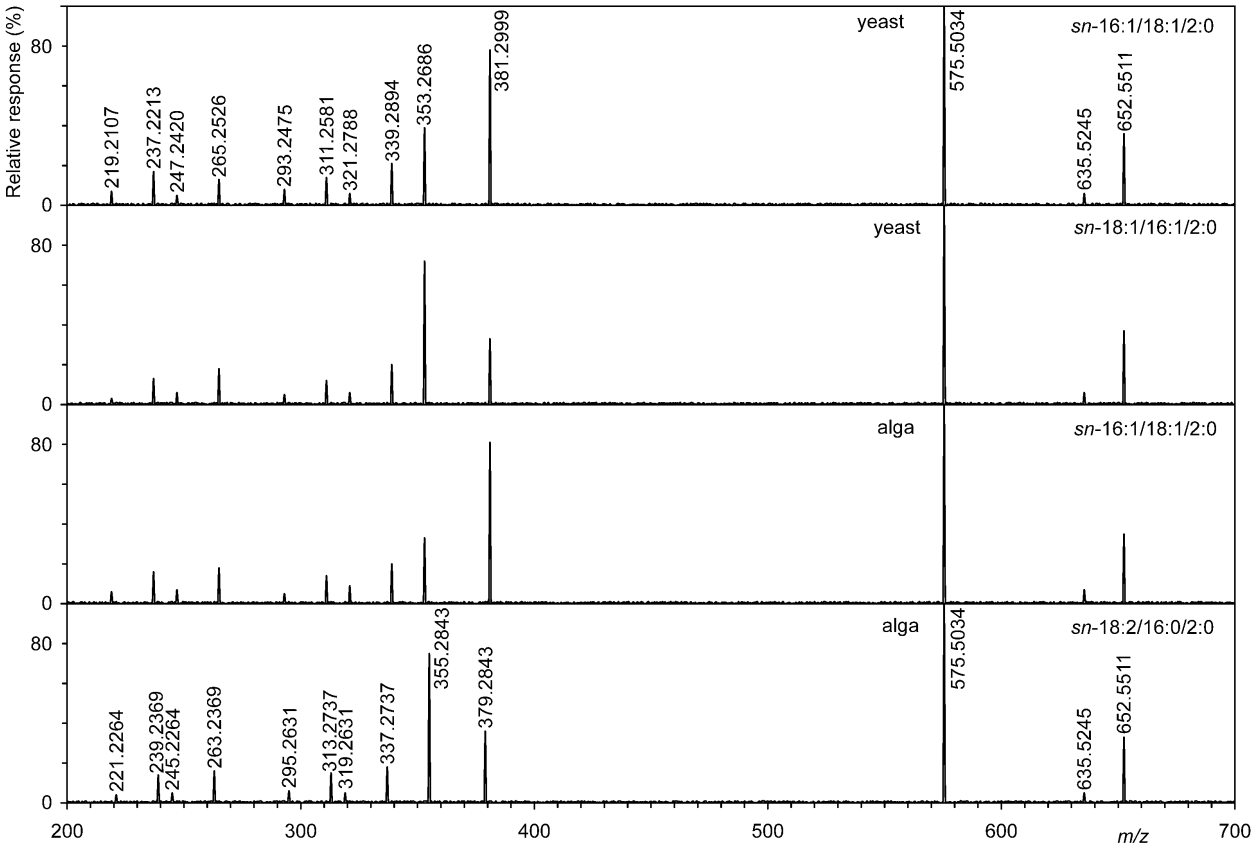


Fig. 4s. The tandem mass spectra of three AcTAGs obtained from molecular species of 16:1/18:1/18:1/16:1-CL (yeast) and 16:1/18:1/18:2/16:0-CL (alga).

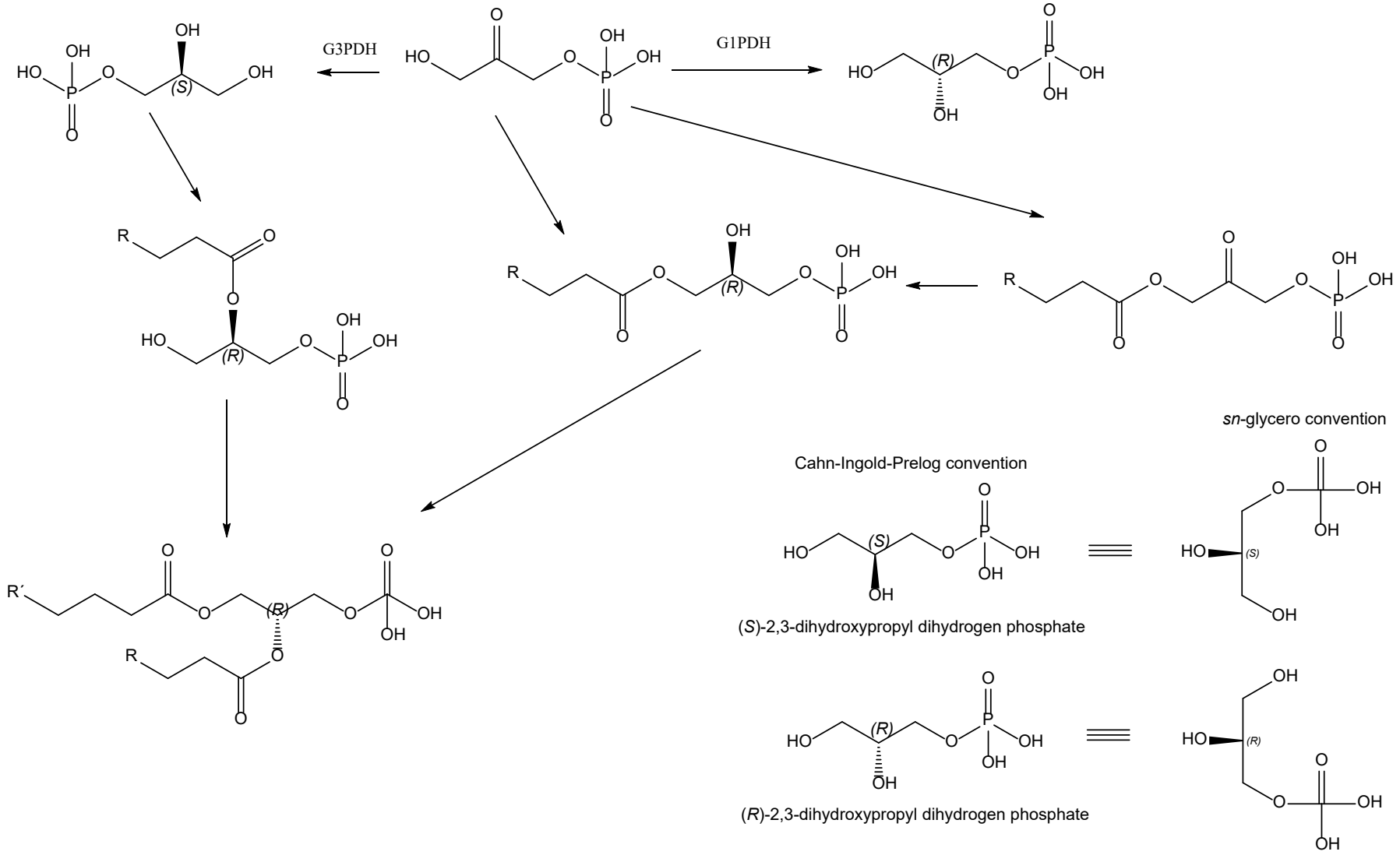


Fig. 5S. Biosynthesis of phosphatidic acid. Both enantiomers and their *sn*-glycerol and Cahn–Ingold–Prelog nomenclatures, *sn*-glycerol-1-phosphate dehydrogenase (G1PDH), *sn*-glycerol-3-phosphate dehydrogenase (G3PDH).