Supplements

Lipidomic analysis of diatoms cultivated with silica nanoparticles

Tomáš Řezanka[a,*], Michal Řezanka[b], Dana Mezricky[c], Milada Vítová[d]

[a] Institute of Microbiology of the Czech Academy of Sciences, Vídeňská 1083, 142 20 Prague, Czech Republic

[b] Department of Nanochemistry, Institute for Nanomaterials, Advanced Technologies and Innovation, Technical University of Liberec, Studentská 1402/2, 461 17 Liberec 1, Czech Republic

[c] Medical and Pharmaceutical Biotechnology, IMC University of Applied Sciences, Piaristengasse1, 3500 Krems, Austria

[d] Institute of Microbiology of the Czech Academy of Sciences, Centre Algatech, Laboratory of Cell Cycles of Algae, Novohradská 237, 379 81 Třeboň, Czech Republic

Results and Discussion

SQDG was identified by a negative ESI because it contains a sulfo group, and this group provides a strong negative charge. From the tandem mass spectrum follows, see Fig. 6, that the ion $[M-H]^-$ shows *m/z* at 839.4985. This ion is cleaved with the concomitant loss of carboxylate anion $[R_2COO]^-$ at *m/z* 255.2324 (*sn*-2, palmitic acid) and at the carboxylate anion at *m/z* 301.2173 $[R_1COO]^-$ (*sn*-1, eicosapentaenoic acid). Ions having the structure $[M-R_1COOH-H]^-$) at *m/z* 583.2583 and 537.2739 result the loss of neutral moieties (fatty acids) from the precursor ion ($[M-H]^-$). As with other glycosyldiacylglycerols (e.g. MGDG or DGDG), an intense ion having a carbohydrate structure at *m/z* 225.0075 ($[C_6H_9O_7S]^-$) is present in the spectrum. This ion can be used to identify SQDG by the selected ion monitoring (SIM) method, see Fig. 3. To identify the esterified acid at the *sn*-1 or *sn*-2 position, the rule that the *sn*-2 lyso lipid is more stable (has a higher abundance, i.e. 42 versus 28 relative%, see Fig. 6, can be advantageously used, and therefore palmitic acid is esterified at the *sn*-2 position. The resulting structure of SQDG was therefore designated as 20:5/16:0-SQDG.

Materials and methods

Synthesis of 1-stearoyl-2-arachidonoyl-sn-3-phosphatidyl sulfocholine

18:0/20:4 PSC was synthesized from dimethylsulfocholine, prepared by procedure previously described [1], i.e. reaction of bromoethanol and dimethyl sulfide. Further, 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphate (18:0/20:4 PA, commercially obtained from

Sigma) and dimethylsulfocholine bromide were stirred overnight at room temperature [2]. The reaction mixture was purified by TLC, see Experimental.

Isolation of lipids

The extraction procedure was based on the method of [3], except that propan-2-ol was substituted for methanol, since propan-2-ol does not serve as a substrate for phospholipases [4]. The alcohol-water mixture was cooled, one part chloroform was added and the lipids from the lyophilized cells of different cultivations of *G. sulphuraria* were extracted for 30 min. Insoluble material was sedimented by centrifugation and the supernatant was separated into two phases. The aqueous phase was aspirated off and the chloroform phase was washed three times with two parts 1 M KCl each. The resulting chloroform phase was evaporated to dryness under reduced pressure.

Total lipid extracts were applied to Sep-Pak Vac Silica cartridge 35cc (Waters; with 10 grams of normal-phase silica), and lipids were subsequently eluted from the cartridge with 40 mL of chloroform-methanol (2:1). The eluate of lipids was evaporated and the oil samples were dissolved in an acetonitrile-propan-2-ol-hexane mixture (1:1:1, v/v/v), which was injected for HPLC analysis.

FAMEs and 3-pyridylcarbonyl esters analysis

The lipids (~5 mg) were saponified overnight in 10% KOH-MeOH at room temperature. A fatty acid fraction obtained from saponification was partitioned between alkali solution (pH 9) and Et_2O to remove basic and neutral components. The aqueous phase, containing fatty acids, was acidified to pH 2 and extracted with hexane. The fatty acid fraction was methylated using CH_2N_2. GC-MS of fatty acid methyl ester (FAME) mixture was done on a Finnigan 1020 B in EI mode. 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. The structures of FAMEs were confirmed by comparison of retention times and fragmentation patterns with those of the standard FAMEs (Supelco, Prague) [5,6].

A solution of potassium *tert*-butoxide in tetrahydrofuran (0.5 mL, 1.0 M) was added to nicotinyl alcohol (1 mL). After mixing, the appropriate fatty acid methyl esters (~0.5 mg) in dry dichloromethane (1 mL) were added, and the mixture was held at 40 °C for 30 min in a

closed vial. After cooling to room temperature, water and hexane were added, and the organic phase was collected, dried over anhydrous sodium sulfate, and evaporated.

A fatty acid 3-pyridylcarbonyl ester mixture was analyzed on the instrument described above. Injection temperature (splitless injection) was 100 °C, and an Ultra-1 capillary column (Supelco, Czech Republic) 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 *m/z* 50-500.

Lipidomic analysis by high-resolution electrospray ionization mass spectrometry and HILIC-LC/MS-ESI

The HPLC equipment consisted of a 1090 Win system, PV5 ternary pump and automatic injector (HP 1090 series, Hewlett Packard, USA), and Ascentis® Express OH5, UHPLC HILIC column (2.7 µm particle size, L × I.D. 15 cm × 2.1 mm) (Supelco, Prague, Czech Republic) with an injection volume of 10 µL. UHPLC was performed at a flow rate of 0.33 mL/min for the lipids from all cultivations, with a linear gradient from mobile phase containing methanol/acetonitrile/aqueous 1 mM ammonium acetate (50:30:20, v/v/v) to methanol/acetonitrile/aqueous 1 mM ammonium acetate (10:70:20, v/v/v) for 60 min. Column temperature was 35 °C and a re-equilibration period was 30 min between runs. The whole UHPLC flow was introduced into the ESI source without any splitting.

LTQ Orbitrap Velos (Thermo Fisher Scientific, Bremen, Germany), a high resolution hybrid mass spectrometer equipped with a heated electrospray interface (HESI) was used. ESI-MS analysis was performed in the FT positive ion mode. MS spectra were acquired with target mass resolution of R = 105,000 at *m/z* 800. The ion spray voltage was set at 3.5 kV and the scan range of the instruments was set at *m/z* 200–1500. Nitrogen was used as a nebulizer gas – set at 18 arbitrary units (sheath gas) and 7 arbitrary units (auxiliary gas). Helium was used as a collision gas for CID experiments. The CID normalization energy of 35% was used for the fragmentation of parent ions. The MS/MS product ions were detected by the high resolution FT mode. Calibration was done with the use of Pierce Negative Ion Calibration solution (Thermo Scientific). Lock mass was used (stearic acid *m/z* 283.26425) by the ESI negative MS spectra acquisition. Isotope pattern model of the suggested ion elemental composition was then compared to the measured one to verify the final ion elemental composition. Further source conditions in the positive mode: vaporizer temperature, 250°C; capillary temperature, 230°C; capillary voltage, 50 V; tube lens voltage, 170 V.

The same apparatus was used for negative ion mode. MS spectra were acquired with target mass resolution (full width at half maximum [FWHM]) 110,000 at *m/z* 800. This value is sufficient, for example, for the complete separation of $[M+Li]^+$ ions at *m/z* 789.5075 (16:0/20:5-PSC) and $[M+Li+2]^+$ ions at *m/z* 789.4985 (16:1/20:5-PSC). The difference was 0.009 Da, and the required FWHM would be at *m/z* 800 of about 88,000.

The ion spray voltage was set at -2500V and the scan range of the instruments was set at *m/z* 200-1500. Nitrogen was used as a nebulizer gas - set at 18 arbitrary units (sheath gas) and 7 arbitrary units (aux gas). Helium was used as a collision gas for CID experiments. The CID normalization energy of 35% was used for the parent ions fragmentation. The MS/MS product ions were detected by the high resolution FT mode. The H-ESI temperature was set to 250 °C.

Mass accuracy (determined via the lock mass) was under 1 ppm. The elemental composition of the ions of interest was determined using the Xcalibur 2.2 software (Thermo Scientific). Even electron rule was used and the maximum mass accuracy was set to 1 ppm.

Flow Injection Analysis (FIA) was used for sample lipidomic analysis of introduction into the heated ESI (H-ESI) ion source. Acetonitrile/water (50/50 v/v) was used at the flow rate of 150 μL/min. The H-ESI temperature was set to 250 °C.

References

[1] A.G. Howard, D.W. Russel, Borohydride-coupled HPLC-FPD instrumentation and its use in the determination of dimethylsulfonium compounds, Anal Chem, 69 (1997) 2882-2887.

[2] P.A. Tremblay, M. Kates, Chemical synthesis of sn-3-phosphatidyl sulfocholine, a sulfonium analog of lecithin, Can J Biochem, 57 (1979) 595-604.

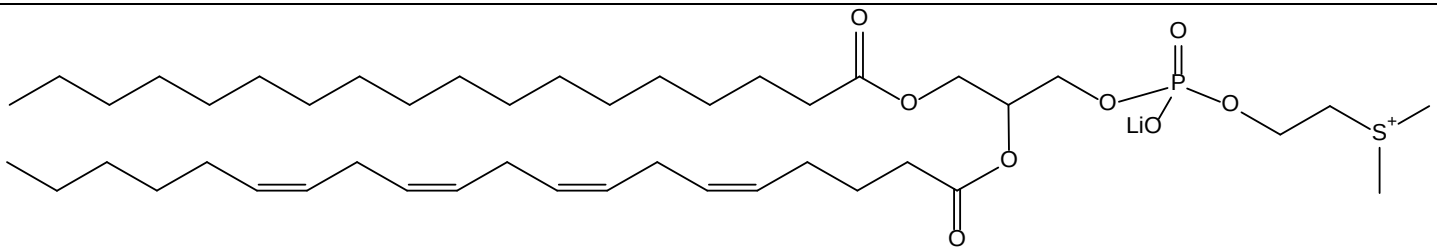
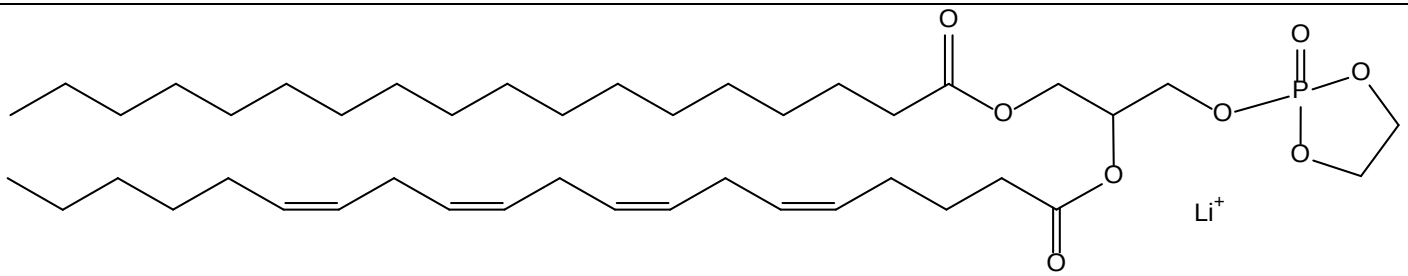
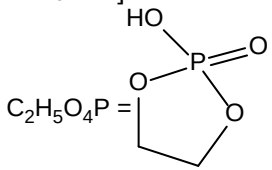
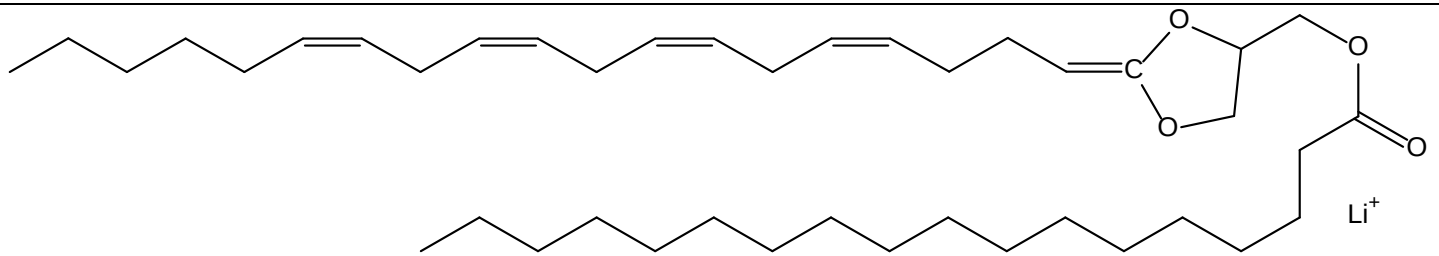
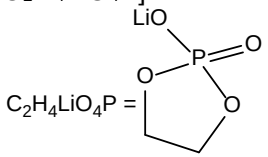
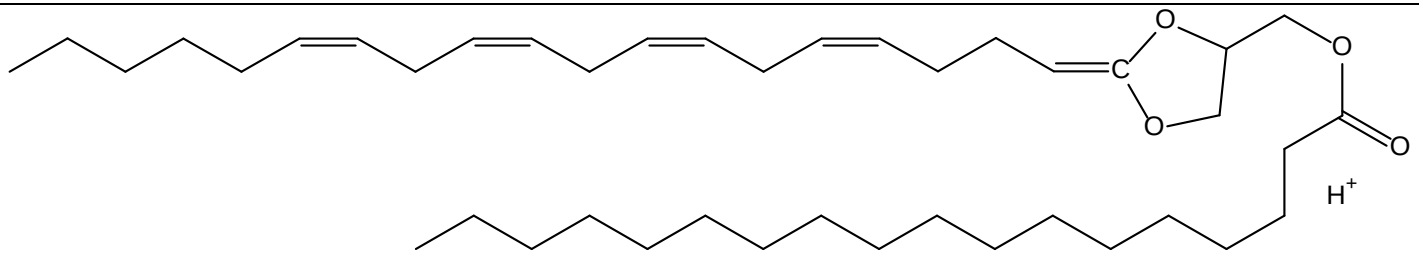
[3] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification, Can J Biochem Physiol, 37 (1959) 911-917.

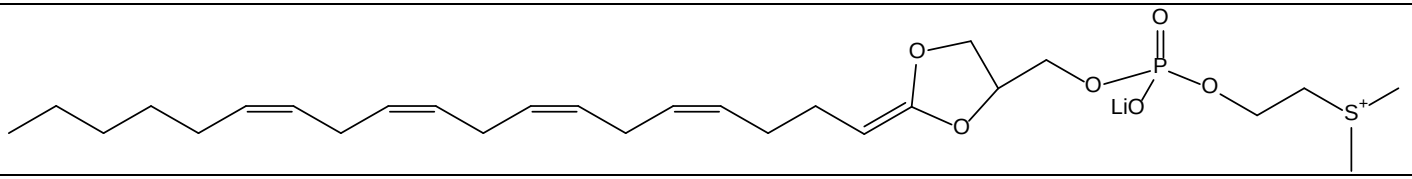
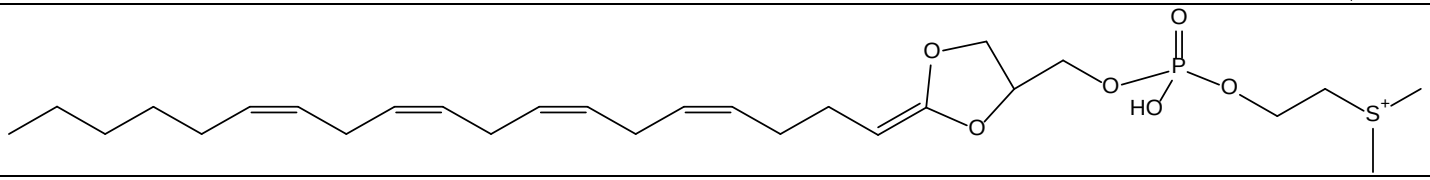
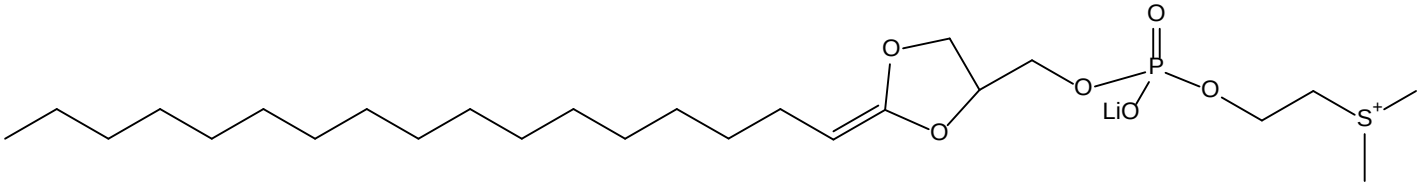
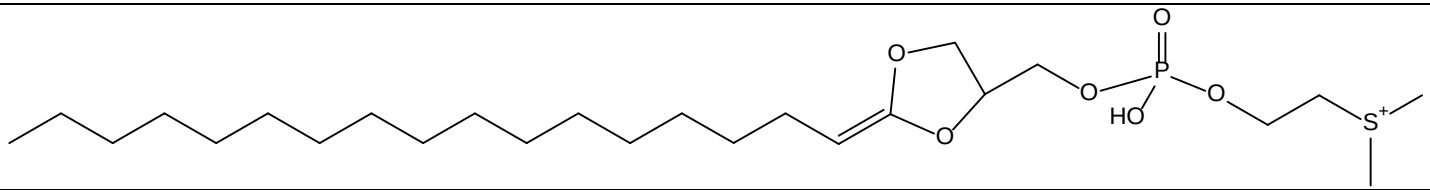
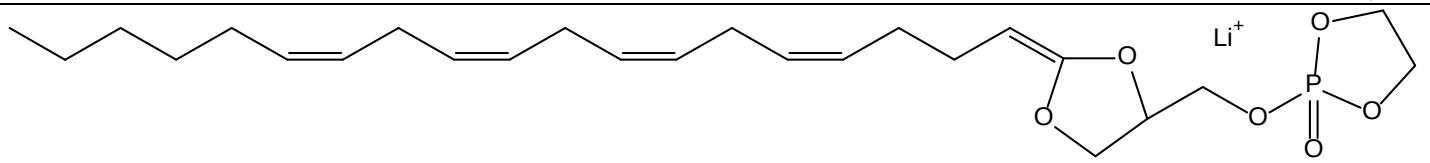
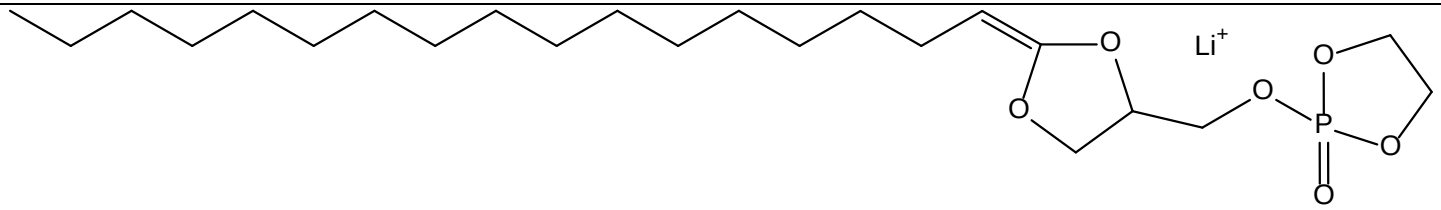
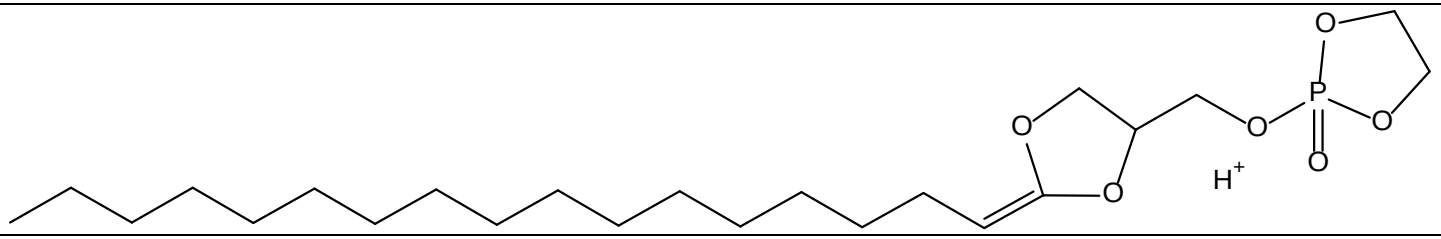
[4] M. Kates, B.E. Volcani, Biosynthetic pathways for phosphatidylsulfocholine, the sulfonium analogue of phosphatidylcholine, in diatoms. In: Kien, R.P., Visscher, P.T., Keller, M.D., Kirst, G.O. (Eds.), Biological and environmental chemistry of DMSP and related sulfonium compounds. Plenum Press, New York, (1996) pp. 109-119.

[5] T. Rezanka, Identification of very long polyenoic acids as picolinyl esters by Ag+ ion-exchange high-performance liquid-chromatography, reversed-phase high-performance liquid-chromatography and gas-chromatography mass-spectrometry, J Chromatogr, 513 (1990) 344-348.

[6] A. Vancura, T. Rezanka, J. Marslalek, K. Melzoch, G. Basarova, V. Kristan, Metabolism of L-threonine and fatty-acids and tylosin biosynthesis in *Streptomyces fradiae*, FEMS Microbiol Lett, 49 (1988) 411-415.

Table 1S. Tandem MS of 18:0/20:4-PSC.

Abbreviation	m/z	abundance	structure
$[M+Li]^+$	819.5544	21	
$[M+Li-62]^+$ $[M+Li-S(CH_2)_2]^+$	757.5354	11	
$[M+Li-186]^+$ $[M+Li-S(CH_2)_2-C_2H_5O_4P]^+$ 	633.5429	100	
$[M+Li-192]^+$ $[M+Li-S(CH_2)_2-C_2H_4LiO_4P]^+$ 	627.5347	37	

$[M+Li-R_1COOH]^+$	535.2829	10	
$[M+H-R_1COOH]^+$	529.2747	7	
$[M+Li-R_2COOH]^+$	515.3142	6	
$[M+H-R_2COOH]^+$	509.3060	8	
$[M+Li-62-R_1COOH]^+$ $[M+Li-S(CH_3)_2-R_1COOH]^+$	**473.2639**	**19**	
$[M+Li-62-R_2COOH]^+$ $[M+Li-S(CH_3)_2-R_2COOH]^+$	**453.2952**	**7**	
$[M+H-62-R_2COOH]^+$ $[M+H-S(CH_3)_2-R_2COOH]^+$	447.2870	3	

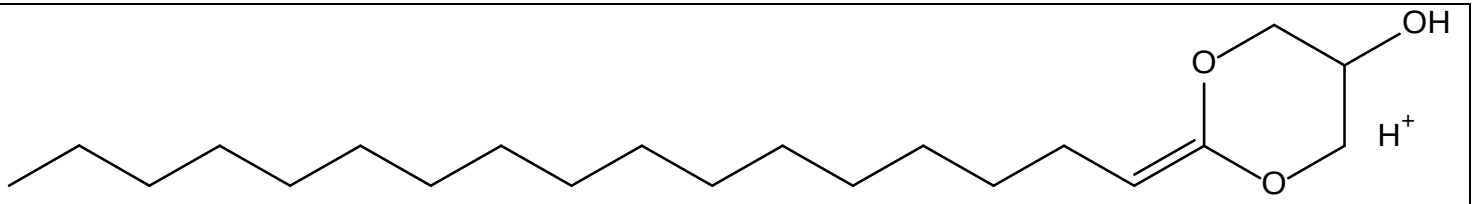
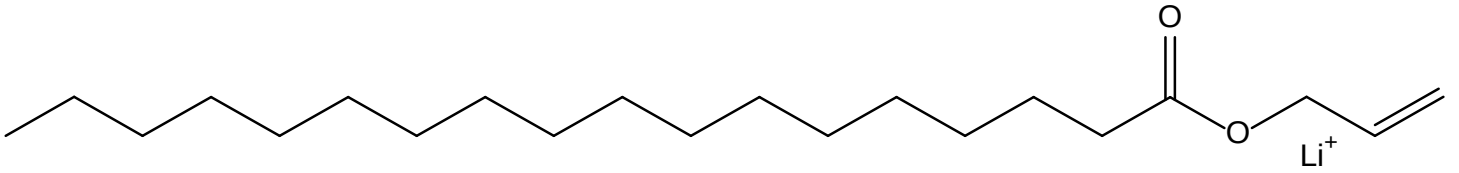
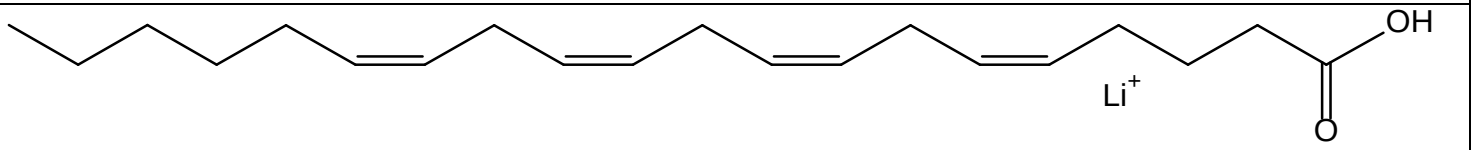
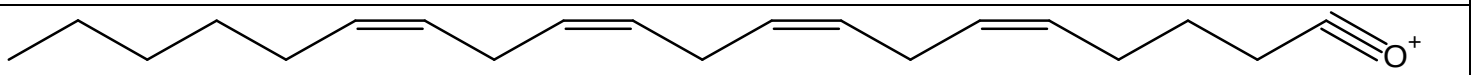
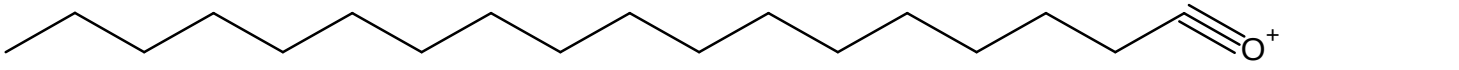
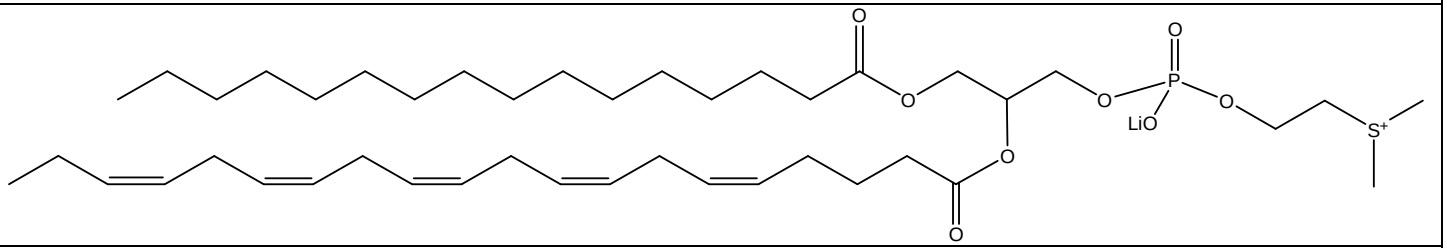
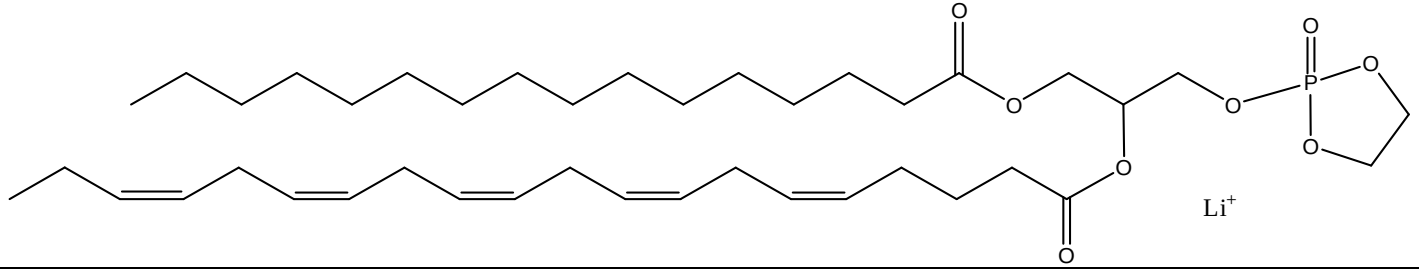
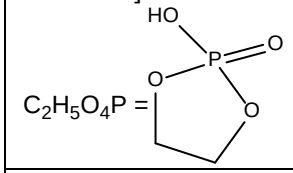
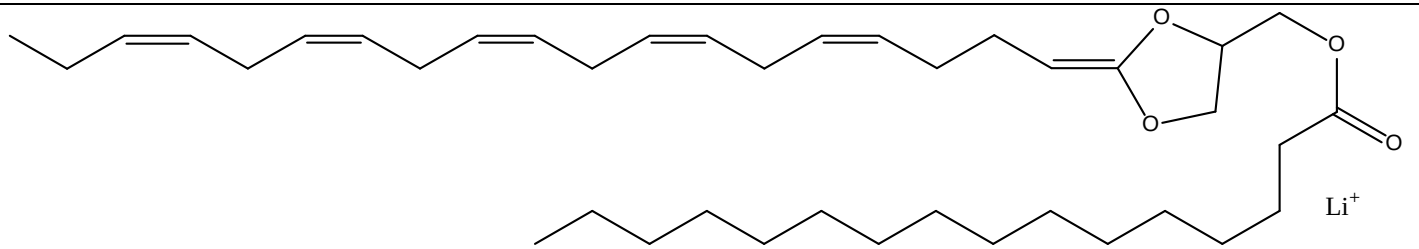
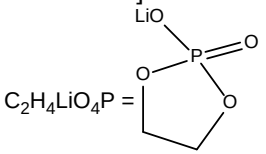
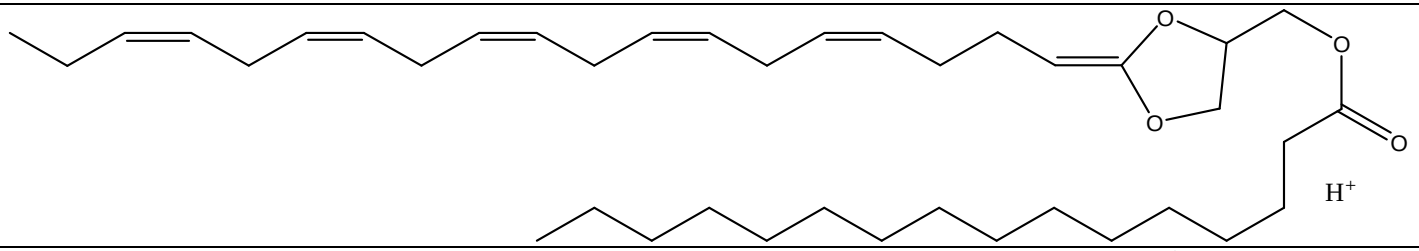
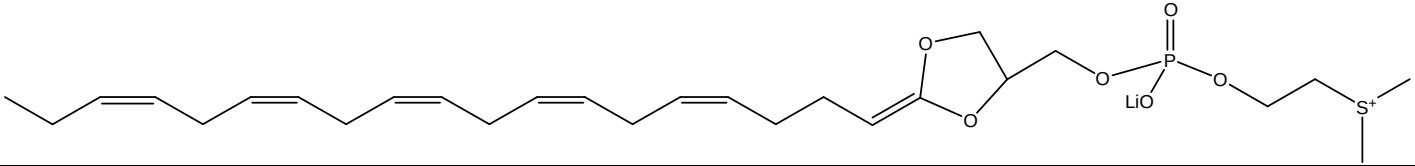
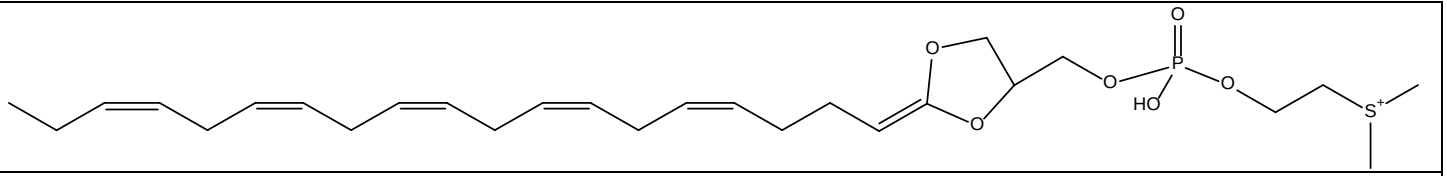
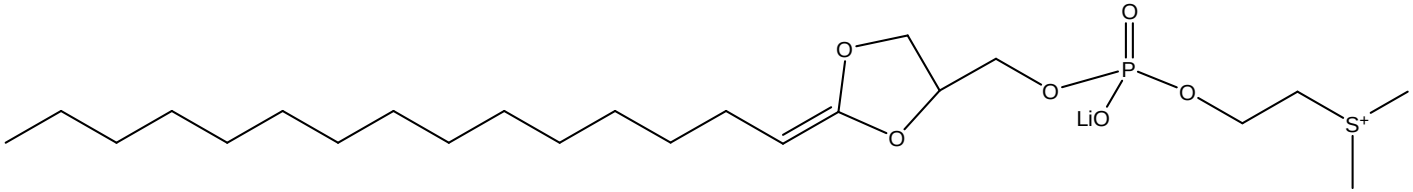
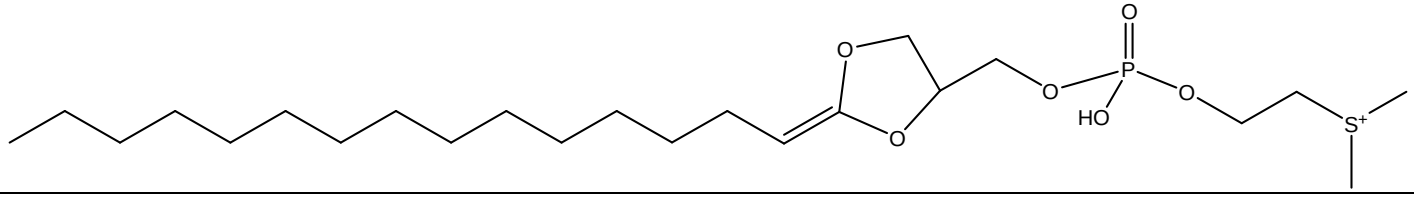
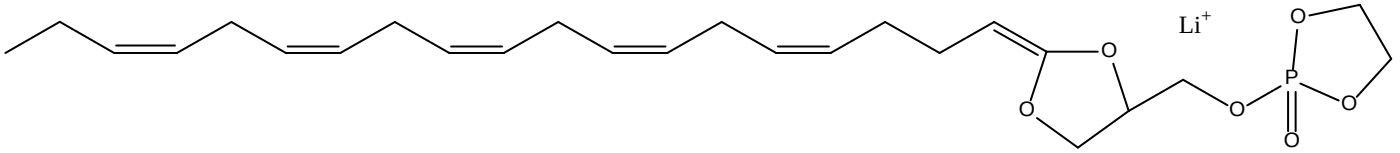
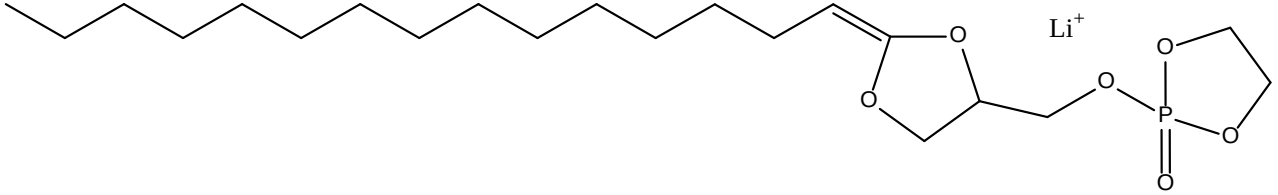
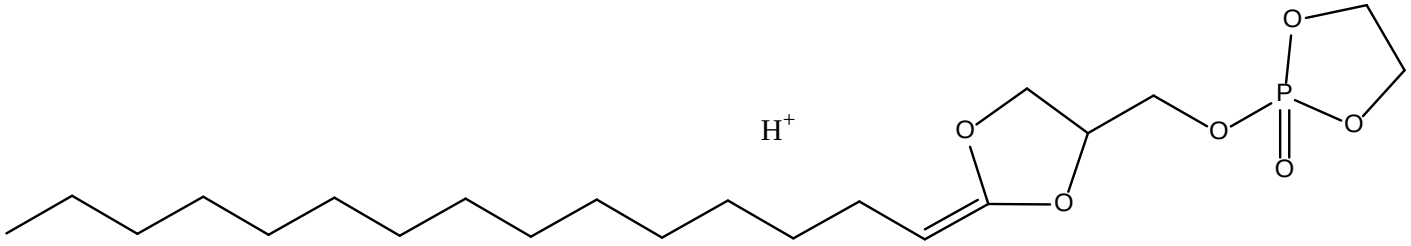
[M+Li-192- $C_{18}H_{27}CH=C=O]^+$ [M+Li-$S(CH_2)_2$- $C_2H_5O_4P$- $C_{18}H_{27}CH=C=O]^+$	341.3050	7	
[M+Li-186- $R_2''CH=CHCOOH]^+$ [M+Li-$S(CH_2)_2$- $C_2H_5O_4P$- $R_2''CH=CHCOOH]^+$	331.3183		
$[R_2COOH+Li]^+$	311.2557		
$[R_2CO]^+$	287.2369		
$[R_1CO]^+$	267.2682		

Table 2S. Tandem MS of 16:0/20:5-PSC.

Abbreviation	m/z	abundance	structure
$[M+Li]^+$	789.5075	19	
$[M+Li-62]^+$ $[M+Li-S(CH_2)_2]^+$	727.4885	8	
$[M+Li-186]^+$ $[M+Li-S(CH_2)_2-C_2H_5O_4P]^+$ 	603.4959	100	
$[M+Li-192]^+$ $[M+Li-S(CH_2)_2-C_2H_4LiO_4P]^+$ 	597.4877	31	
$[M+Li-R_1COOH]^+$	533.2673	8	

[M+H-R_1COOH]$^+$	527.2591	6	
[M+Li-R_2COOH]$^+$	487.2829	5	
[M+H-R_2COOH]$^+$	481.2747	7	
[M+Li-62-R_1COOH]$^+$ [M+Li-S(CH_3)$_2$-R_1COOH]$^+$	**471.2482**	**15**	
[M+Li-62-R_2COOH]$^+$ [M+Li-S(CH_3)$_2$-R_2COOH]$^+$	**425.2639**	**6**	
[M+H-62-R_2COOH]$^+$ [M+H-S(CH_3)$_2$-R_2COOH]$^+$	419.2557	3	

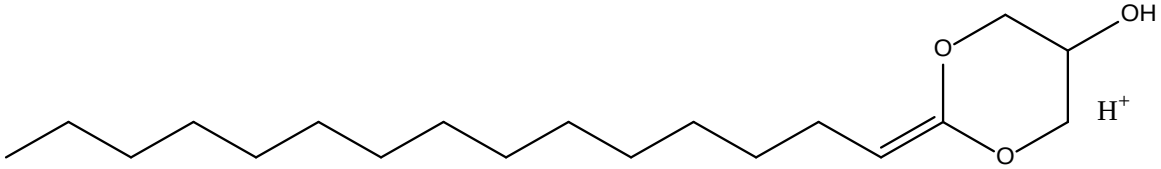
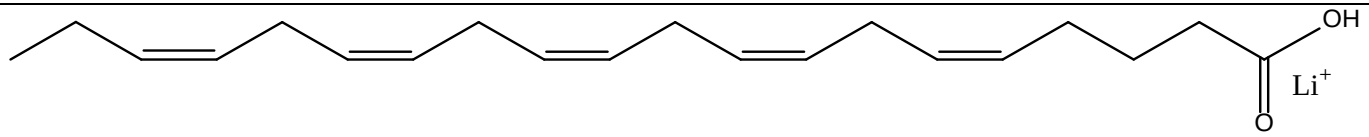
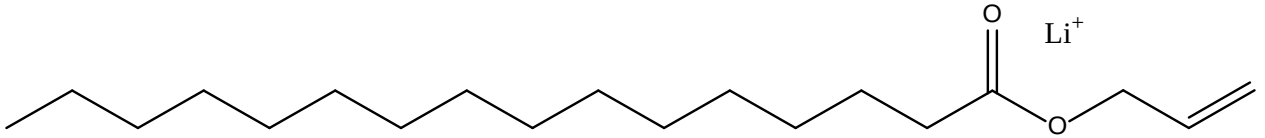
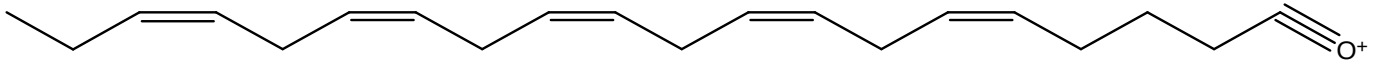
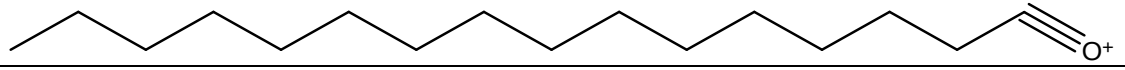
[M+Li-192- $C_{18}H_{27}CH=C=O]^+$ [M+Li-$S(CH_2)_2$- $C_2H_5O_4P$- $C_{18}H_{27}CH=C=O]^+$	313.2737	7	
$[R_2COOH+Li]^+$	309.2401	5	
[M+Li-186- $R_2´´CH=CHCOOH]^+$ [M+Li-$S(CH_2)_2$- $C_2H_5O_4P$- $R_2´´CH=CHCOOH]^+$	303.2870	9	
$[R_2CO]^+$	285.2213	2	
$[R_1CO]^+$	239.2369	2	

Fig. 1S. Heat maps showing the abundance of both lipids that contain sulfolipids (SQDG and PSC), classified based on polar head type in the three strains of diatoms. The chromatogram is shown in Figure 3.

Strain	SQDG	PSC	Relative %
Diadesmis gallica 766 control 1.0 mM sodium metasilicate			100[a]
Diadesmis gallica 766 0.5 mM nano-SiO2			110
Diadesmis gallica 766 1.0 mM nano-SiO2			120
Diadesmis gallica 766 2.0 mM nano-SiO2			130
Diadesmis gallica 765 control 1.0 mM sodium metasilicate			140
Diadesmis gallica 765 0.5 mM nano-SiO2			150
Diadesmis gallica 765 1.0 mM nano-SiO2			160
Diadesmis gallica 765 2.0 mM nano-SiO2			
Navicula atomus 383 control 1.0 mM sodium metasilicate			
Navicula atomus 383 0.5 mM nano-SiO2			

[a] control, i.e. cultivation with 1.0 mM sodium metasilicate was taken as 100%