Supplements

2.2. DNA extraction, PCR and sequencing

Total genomic DNA was extracted from algal cultures following the standard protocol in DNeasy Plant Mini Kit (Qiagen, Germany), with minor modifications: At the beginning of the procedure the cells were mechanically disrupted by shaking for 15 min (30 Hz) in the presence of glass beads (3 mm diameter, Sigma-Aldrich, Czech Republic) in the Mixer Mill MM 400 (Retsch, Germany). Afterwards, quality and concentration of DNA was checked on the NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific, USA).

The internal transcribed spacer region 2 (ITS2 rDNA) was amplified from DNA isolates by PCR using the primers TW-81 (5′- GGGATCCGTTTCCGTAGGTGAACCTGC-3′), AB-28 (5′- GGGATCCATATGCTTAAGTTCAGCGGGT-3′) [29], Al1500af (5′- GCGCGCTACACTGATGC-3′) [30] and LR3 (5′- GGTCCGTGTTTCAAGACGG-3) [31]. Amplification reactions were performed using the following cycle parameters: 5 min hot start at 97 °C, followed by 37 cycles (1:25 min at 95 °C, 2 min at annealing temperature of 56 °C, 4 min at 72 °C) and 7 min at 72 °C. Each 35 µl PCR reaction contained 1 µl of DNA isolate (diluted to a concentration of 5 ng/µl), 1.4 µl of each 10 µM primer, 2.8 µl of 25 mM $MgCl_2$, 2.6 µl of 2 mM dNTPs, 3.5 µl of 10x Taq buffer + KCl-$MgCl_2$, 22.2 µl sterile Milli-Q water, and 0.1 µl of 5U/µl Taq DNA polymerase (Fermentas, USA).

The PCR products were stained with bromophenol loading dye, quantified on 1.5 % agarose gel and stained with GelRed. The amplification products were purified and sequenced using an Applied Biosystems (Foster City, USA) automated sequencer (ABI 3730xl) at Macrogen (Seoul, Korea). New sequences are available in the NCBI Nucleotide Sequence Database under accession numbers KY311560 and KY311561.

2.4. Isolation of lipids

The extraction procedure was based on the method of [36], except that 2-propanol was substituted for methanol, since 2-propanol does not serve as a substrate for phospholipases [37]. 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 TAGs were subsequently eluted from the cartridge with 40

mL of chloroform-methanol (9:1). Further, polar lipids were eluted from the cartridge by a mixture of 50 ml of methanol acidified with 0.1% of formic acid. The eluate of polar lipids was evaporated and the oil samples were dissolved in an acetonitrile-2-propanol-hexane mixture (1:1:1, v/v/v), which was injected for HPLC analysis.

2.5. FAMEs analysis

The lipids (both TAG and polar 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) [53,54].

2.6. Lipidomic analysis by high-resolution electrospray ionization mass spectrometry

The high resolution hybrid mass spectrometer LTQ Orbitrap Velos (Thermo Scientific, FL, USA) was used. ESI–MS analysis was performed in the positive ion mode. MS spectra were obtained by the Fourier transform mode and were acquired with target mass resolution of R = 30,000 at *m/z* 400 (lock mass 413.6662 Da). 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). Orbitrap resolving power was 30000 full width at half maximum (FWHM). 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. Mass accuracy (controlled via the lock mass) was -1.5 ppm. The elemental composition of the ions of interest was determined with the use of Xcalibur 2.2 software (Thermo Scientific). Even electron rule was used and maximum mass accuracy was set up to 3 ppm. Isotope pattern model of the suggested ion elemental composition was then compared to the measured one to verify the final ion elemental composition. Helium was used as a collision gas for collision-induced dissociation (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 low resolution FT mode. Flow Injection Analysis (FIA) was used for sample 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.

2.7. 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 polar lipids from all four cultivations (pH 1, 2, 3, and 4), 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 or with a linear gradient from mobile phase containing hexane/2-propanol/formic acid/24% aqueous ammonium (80:19:0.5:0.5, v/v/v/v) to 2-propanol/water/formic acid/24% aqueous ammonium 90:9:0.5:0.5 (for separation of DGTA and DGTS, respectively) for 50 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. 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 of R = 30,000 at *m/z* 400. The ion spray voltage was set at -2500V and the scan range of the instruments was set at *m/z* 200-2000. 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.

References not listed in the main text

[53] T. Řezanka, Identification of very long polyenoic acids as picolynyl 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.

[54] T. Řezanka, Polyunsaturated and unusual fatty acids from slime molds, Phytochemistry 33 (1993) 1441–1444.

Table 1S. Composition of stock solutions for preparation of Z8 medium according to Zehnder (Staub 1961): To a 700 mL of distilled water add 10 ml each of stock solutions of macroelements, 0.2 mL Fe-EDTA solution, 0.08 mL of Gaffron solution of micronutrients and make up to 1 L with distilled water. For a large-scale platform cultivation (a 150 L volume), weighed chemicals can be poured directly onto the platform and allowed to dissolve with a flow of CO_2.

Medium Z8

Compound	**Stock solution ($g.l^{-1}$ dH_2O)**	**Volume per 1 liter**
$NaNO_3$	46.7	10 mL
$Ca(NO_3)_2.4H_2O$	5.9	10 mL
K_2HPO_4	3.1	10 mL
$MgSO_4.7H_2O$	2.5	10 mL
Na_2CO_3	2.1	10 mL
Fe-EDTA solution	See the prescription below	0.2 mL
Gaffron micronutrient solution	See the prescription below	0.08 mL

Fe-EDTA solution

Compound	**Amount**
HCl (35%)	2.2 mL
dH_2O	250 mL
$FeCl_3.6H_2O$	4.5 g
Na_2EDTA	4.65 g

Gaffron micronutrient solution

Compound	**Amount per 100 ml**
H_3BO_3	0.31 g
$MnSO_4.4H_2O$	0.223 g
$Na_2WO_4.2H_2O$	0.003 g
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.0088 g
KBr	0.0119 g
KI	0.0083 g
$ZnSO_4.7H_2O$	0.0287 g
$Cd(NO_3)_2.4H_2O$	0.0154 g
$Co(NO_3)_2.6H_2O$	0.0146 g
$CuSO_4.5H_2O$	0.0125 g
$NiSO_4(NH_4)_2SO_4.6H_2O$	0.0198 g
$Cr(NO_3)_2.7H_2O$	0.0037 g
$V_2O_4(SO_4)_3.16H_2O$	0.0035 g
$Al_2(SO_4)_3K_2SO_4.24\ H_2O$	0.0474 g

Table 2S. Fatty acid composition from three parallel cultivations (a–c) of the strains CCALA 1082 and CCALA 1084.

FA	Abbreviations	strain1082a	±S.D.[a]	strain1082b	±S.D.	strain1082c	±S.D.	strain1084a	±S.D.	strain1084b	±S.D.	strain1084c	±S.D.
14:0	M	0.041	0.002	0.041	0.001	0.042	0.001	0.134	0.001	0.115	0.002	0.089	0.001
14:1	Mo	0.047	0.003	0.038	0.002	0.027	0.001	0.090	0.003	0.083	0.001	0.116	0.002
16:0	P	14.026	0.012	13.865	0.009	14.058	0.011	13.685	0.012	13.962	0.010	14.542	0.016
16:1n-11	He	4.669	0.006	4.864	0.005	4.765	0.004	3.241	0.004	3.179	0.003	3.227	0.004
16:1n-7	Po	0.167	0.001	0.157	0.002	0.184	0.002	0.526	0.003	0.534	0.001	0.475	0.001
16:2n-6	Pl	1.695	0.004	1.663	0.005	1.737	0.006	1.755	0.007	1.841	0.002	1.753	0.002
16:3n-3	Pn	4.042	0.008	4.129	0.006	3.946	0.007	3.478	0.009	3.542	0.003	3.486	0.004
18:0	S	1.718	0.004	1.674	0.005	1.669	0.003	2.247	0.008	2.158	0.003	2.238	0.002
18:1n-9	O	6.654	0.007	6.459	0.006	6.586	0.005	12.164	0.013	12.748	0.009	12.841	0.008
18:1n-7	V	5.109	0.006	5.049	0.007	4.900	0.008	2.238	0.005	2.244	0.004	2.159	0.003
18:2n-6	L	13.265	0.011	13.064	0.009	13.369	0.010	16.361	0.014	16.369	0.012	16.264	0.010
18:3n-6	γ-Ln	1.457	0.002	1.458	0.002	1.543	0.002	1.572	0.004	1.571	0.006	1.562	0.004
18:3n-3	Ln	10.441	0.015	10.342	0.012	10.068	0.013	8.512	0.007	8.475	0.007	8.541	0.004
20:2n-6	Ad	0.014	0.001	0.047	0.002	0.012	0.002	0.245	0.003	0.183	0.002	0.236	0.001
20:3n-6	At	1.213	0.004	1.152	0.003	1.232	0.003	0.879	0.002	0.944	0.002	0.948	0.004
20:4n-6	A	33.752	0.016	34.233	0.013	34.071	0.012	30.782	0.015	29.982	0.014	29.459	0.013
20:4n-3	Et	0.037	0.002	0.038	0.002	0.034	0.002	0.694	0.004	0.677	0.003	0.678	0.003
20:5n-3	E	1.653	0.004	1.727	0.003	1.757	0.002	1.397	0.002	1.393	0.003	1.386	0.004

[a] Arithmetic means from three analyses ± standard deviations.

Table 3S. Product ions for molecular species of PE 20:3/20:5.

m/z	Abundance	Ion Description
786.5079	27	Precursor ion $[M-H]^-$
502.2939	25	Loss of *sn*-2 acyl chain as ketene (RCH=C=O) from $[M-H]^-$
498.2626	15	Loss of *sn*-1 acyl chain as ketene (RCH=C=O) from $[M-H]^-$
484.2834	14	Neutral loss of *sn*-2 RCOOH group from $[M-H]^-$
480.2521	8	Neutral loss of *sn*-1 RCOOH group from $[M-H]^-$
305.2486	45	*sn*-1 $RCOO^-$ ion
301.2173	100	*sn*-2 $RCOO^-$ ion
257.2275	16	Loss of CO_2 from *sn*-2 $RCOO^-$ ion (20:5)

Table 4S. Product ions for molecular species of PC 20:3/20:5.

m/z	Abundance	Ion Description
888.5760	35	Precursor ion $[M+acetate]^-$
814.5392	100	Loss of CH_3 and acetate from precursor ion
743.4657	21	Loss of choline and acetate from precursor ion
530.3252	32	Loss of *sn*-2 acyl chain as ketene (RCH=C=O), CH_3 and acetate from precursor ion
526.2939	21	Loss of *sn*-1 acyl chain as ketene (RCH=C=O), CH_3 and acetate from precursor ion
512.3147	23	Neutral loss of *sn*-2 RCOOH group, loss of CH_3 and acetate from precursor ion
508.2834	14	Neutral loss of *sn*-1 RCOOH group, loss of CH_3 and acetate from precursor ion
305.2486	29	*sn*-1 RCOO- ion
301.2173	17	*sn*-2 RCOO- ion
257.2275	14	Loss of CO_2 from *sn*-2 RCOO- ion (20:5)
224.0693	17	Glycerophosphocholine with loss of CH_3 and H_2O

Table 5S. Product ions for molecular species of PI 20:3/20:5.

m/z	Abundance	Ion Description
905.5186	45	Precursor ion $[M-H]^-$
743.4657	6	Loss of inositol from $[M-H]^-$
621.3045	17	Loss of *sn*-2 acyl chain as ketene (RCH=C=O) from $[M-H]^-$
617.2732	7	Loss of *sn*-1 acyl chain as ketene (RCH=C=O) from $[M-H]^-$
603.2940	22	Neutral loss of *sn*-2 RCOOH group from $[M-H]^-$
599.2627	11	Neutral loss of *sn*-1 RCOOH group from $[M-H]^-$
459.2517	5	Loss of *sn*-2 acyl chain as ketene (RCH=C=O) and inositol from $[M-H]^-$
455.2204	6	Loss of *sn*-1 acyl chain as ketene (RCH=C=O) and inositol from $[M-H]^-$
441.2412	5	Neutral loss of *sn*-2 RCOOH group and inositol from $[M-H]^-$
437.2099	7	Neutral loss of *sn*-1 RCOOH group and inositol from $[M-H]^-$
315.0487	6	Glycerophosphoinositol $-H_2O$
305.2486	100	*sn*-1 $RCOO^-$ ion
301.2173	75	*sn*-2 $RCOO^-$ ion
297.0381	12	Glycerophosphoinositol $-2\times H_2O$
259.0225	18	Inositol phosphate ion
257.2275	24	Loss of CO_2 from *sn*-2 $RCOO^-$ ion (20:5)
241.0119	63	Inositol phosphate ion - H_2O
223.0013	18	Inositol phosphate ion – $2\times H_2O$

Table 6S. Product ions for molecular species of Pl-A/PE, Po-E/PE, Ln-Ln/PE, and Pn-At/PE.

Pl-A/	
734.4766	Precursor ion $[M-H]^-$
500.2783	Loss of *sn*-1 acyl chain as ketene (RCH=C=O) from $[M-H]^-$
482.2677	Neutral loss of *sn*-1 RCOOH group from $[M-H]^-$
448.2471	Loss of *sn*-2 acyl chain as ketene (RCH=C=O) from $[M-H]^-$
430.2365	Neutral loss of *sn*-2 RCOOH group from $[M-H]^-$
303.2326	*sn*-2 $RCOO^-$ ion
259.2426	Loss of CO_2 from *sn*-2 $RCOO^-$ ion (PUFA)
251.2021	*sn*-1 $RCOO^-$ ion

Po-E/	
734.4766	Precursor ion $[M-H]^-$
498.2626	Loss of *sn*-1 acyl chain as ketene (RCH=C=O) from $[M-H]^-$
480.2521	Neutral loss of *sn*-1 RCOOH group from $[M-H]^-$
450.2626	Loss of *sn*-2 acyl chain as ketene (RCH=C=O) from $[M-H]^-$
432.2521	Neutral loss of *sn*-2 RCOOH group from $[M-H]^-$
301.2173	*sn*-2 $RCOO^-$ ion
257.2275	Loss of CO_2 from *sn*-2 $RCOO^-$ ion (PUFA)
253.2173	*sn*-1 $RCOO^-$ ion

Ln-Ln/	
734.4766	Precursor ion $[M-H]^-$
474.2626	Loss of acyl chain as ketene (RCH=C=O) from $[M-H]^-$
456.2521	Neutral loss of RCOOH group from $[M-H]^-$
277.2173	$RCOO^-$ ion

Pn-At/	
734.4766	Precursor ion $[M-H]^-$
502.2939	Loss of *sn*-1 acyl chain as ketene (RCH=C=O) from $[M-H]^-$
484.2834	Neutral loss of *sn*-1 RCOOH group from $[M-H]^-$
446.2313	Loss of *sn*-2 acyl chain as ketene (RCH=C=O) from $[M-H]^-$
428.2207	Neutral loss of *sn*-2 RCOOH group from $[M-H]^-$
305.2486	*sn*-2 $RCOO^-$ ion
249.1855	*sn*-1 $RCOO^-$ ion