Localization of 1-deoxysphingolipids to mitochondria induces mitochondrial dysfunction

Supplemental material

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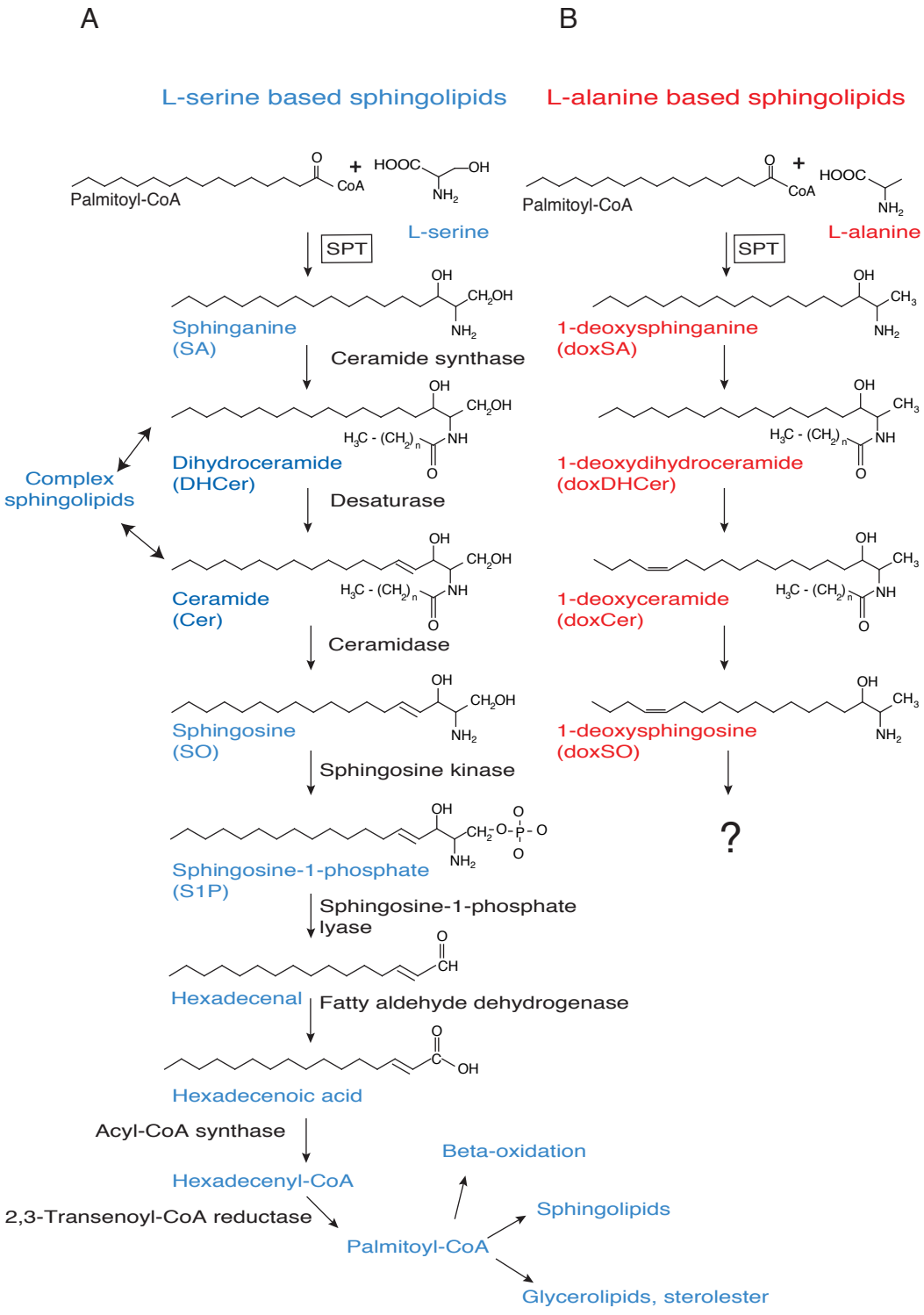
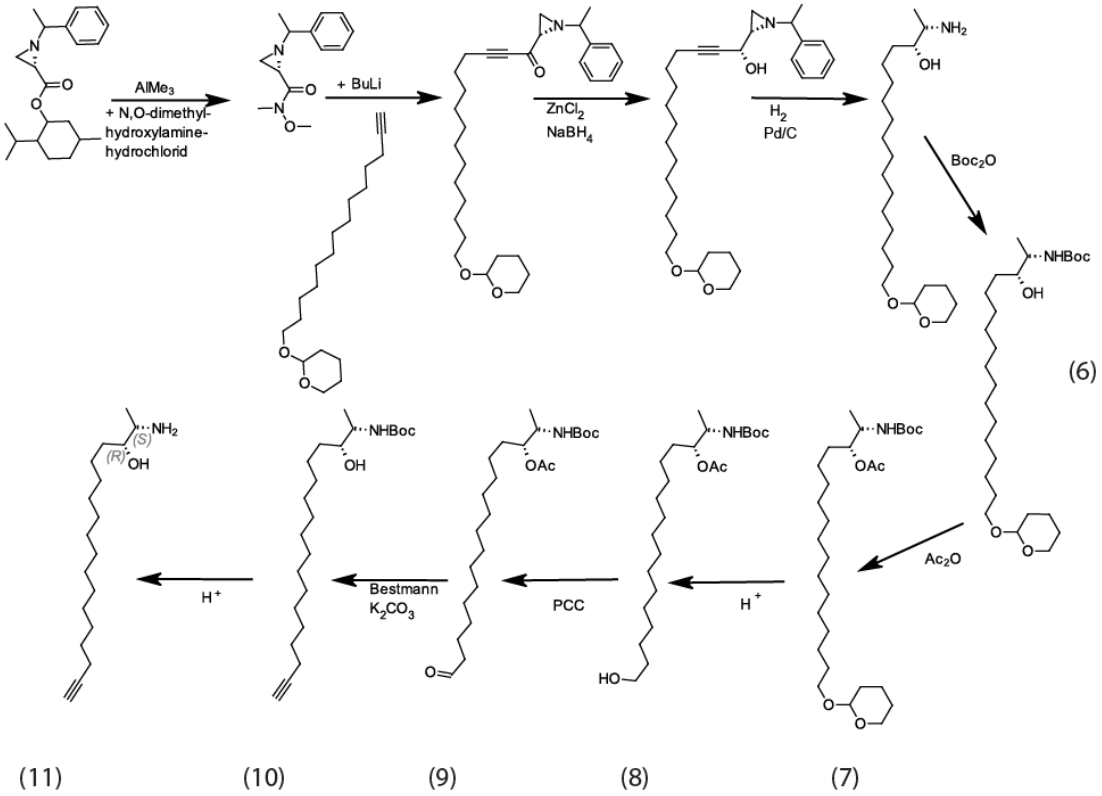

A
B
L-serine based sphingolipids
L-alanine based sphingolipids
Palmitoyl-CoA
L-serine
L-alanine
SPT
Sphinganine
(SA)
1-deoxysphinganine
(doxSA)
Ceramide synthase
Dihydroceramide
(DHCer)
1-deoxydihydroceramide
(doxDHCer)
Complex
sphingolipids
Desaturase
Ceramide
(Cer)
1-deoxyceramide
(doxCer)
Ceramidase
Sphingosine
(SO)
1-deoxysphingosine
(doxSO)
Sphingosine kinase
?
Sphingosine-1-phosphate
(S1P)
Sphingosine-1-phosphate
lyase
Hexadecenal
Fatty aldehyde dehydrogenase
Hexadecenoic acid
Acyl-CoA synthase
Beta-oxidation
Hexadecenyl-CoA
2,3-Transenoyl-CoA reductase
Sphingolipids
Palmitoyl-CoA
Glycerolipids, sterolester

Supplemental Figure S1. Metabolic pathway for canonical sphingolipids (A) and 1-deoxysphingolipids (B).

Serine palmitoyltransferase (SPT) catalyzes the condensation of palmitoyl-CoA with L-serine (panel A) or L-alanine (panel B), leading to the generation of sphinganine (SA) or 1-deoxysphinganine (doxSA), respectively. Ceramide synthases N-acylate the 2-amino position of SA and doxSA to form Dihydroceramides (DHCer) and 1-deoxydihydroceramides (doxDHCer). DHCer desaturase introduces a double bond between C4-C5 in the sphingoid backbone of DHCer, yielding ceramide (Cer). DoxDHCer is also desaturated to 1-deoxyceramide (doxCer), however so far it was not shown whether the same enzyme catalyzes the reaction. Cer are degraded by N-deacylation (ceramidases), leading to the sphingoid bases sphingosine (SO), whereas this reaction has not yet been directly proven for doxCer to 1-deoxysphingosine (doxSO) conversion. SO can be phosphorylated at the C1-hydroxyl group to sphingosine-1-phosphate (S1P), an important signaling lipid. Canonical sphingolipids are degraded and recycled to fatty acids via the sequential action of S1P kinase, S1P lyase, and fatty aldehyde dehydrogenase, resulting in hexadecenoic acid. This fatty acid can be activated by acyl-CoA synthases to the corresponding CoA ester, which is then used for beta-oxidation or lipid synthesis. Because 1-deoxysphingolipids lack the C1-hydroxy group, they are presumably excluded from the described degradation pathway of canonical sphingolipids.

Synthesis of alkyne-doxSA



Supplemental Figure S2. Synthesis of alkyne-doxSA

Preparation of (2):

(2) was synthetized according to Yun et al. (1). 880 mg of (−)-menthyl (S)-1-[(R)-α-methylbenzyl]aziridine-2-carboxylate (Sigma 570516) was dissolved in 10 ml DCM. 4.55 ml 2M $AlMe_3$ in heptane was carefully added under argon at -10ºC. The solution was stirred for 30 min at RT. 1 g of N,O-dimethylhydroxylamine hydrochloride (Sigma D163708) was dissolved in DCM and added dropwise at -10 ºC. The mixture was stirred for 4 hours at RT. The reaction was quenched with water and the organic layer was separated. After extracting with DCM three times, the combined organic phases were dried. Purification by silica gel chromatography (EtOAc: hexane, 9:1) yielded (2) (400 mg, 56%): **1H NMR (400 MHz, $CDCl_3$): 7.4-7.1 (m, 5H), 3,07 (s, 3H), 3.05 (s, 3H), 2.5 (m, 2H), 2.35 (dd, 1H), 1.69 (dd, 1H), 1.41 (d, 3H)**

Preparation of (3):

Synthesis of (3) was performed according to ref. (2). THP-Tetradecynal was synthetized according to ref. (3). 420 mg THP-tetradecyne was dissolved in 3 ml THF at -20 ºC and 1 ml 1.6M n-BuLi in hexane was added dropwise. The solution was stirred for 30 min. Then 400 mg of (2) dissolved in 1 ml THF was added to the reaction. The solution was stirred for 1 h. After addition of 20 ml hexane, 30 ml EtOAc and brine, the organic phase was separated, washed twice with brine, and dried. Purification by silica gel chromatography (hexane:EtOAc, 4:1) yielded (3) (360 mg, 53 %): **1H NMR (400 MHz, $CDCl_3$): 7.4-7.1 (m, 5H), 4.5 (m, 1H), 3.8 (m, 1H), 3.6 (dt, 1H), 3.4 (m, 1H), 3.3 (dt, 1H), 2.55 (q, 1H), 2.36 (dd, 1H), 2.28 (t, 1H), 2.18 (dd, 1H), 1.79 (dd, 1H), 1.5 (m, 10H), 1.38 (d, 3H), 1.34-1.2 (m 16H)**

Preparation of (4):

Stereoselective reduction of the ketone was performed according to ref. (1). 360 mg (3) were dissolved in 4 ml MeOH under argon at -18 ºC. 1.2 ml $ZnCl_2$ (1 M in diethylether) was added and the solution stirred for 1 h. 60 mg $NaBH_4$ was added and the solution was stirred for another hour. Then the reaction was quenched by the addition of water. The product was extracted with DCM (4x 10 ml). The combined organic phases were dried and the product was purified by silica gel chromatography (hexane: EtOAc, 1:1). 216 mg (4) was recovered (yield 60%): **1H NMR (400 MHz, $CDCl_3$): 7.35-7.2 (m, 5H), 4.56 (dd, 1H), 4.28 (m, 1H), 3.85 (m, 1H), 3.71 (m, 1H), 3.48 (m, 1H), 3.36 (m, 1H), 2.6 (q, 1H), 2.1 (dd, 1H), 2.07 (t, 1H), 1.81 (m, 1H), 1.8 (m, 1H), 1.53 (m, 8H), 1.41 (d, 3H), 1.36-1.24 (m, 16H)**

Preparation of (6):

216 mg (4) were dissolved in 5 ml MeOH and reacted with 200 mg Pd and 120 mg $(Boc)_2O$ under 1 atm H_2 at RT for 15 h while stirring. The reaction mixture was filtered and the product purified by silica gel chromatography (hexane: EtOAc, 1:1). 182 mg of (6) were recovered (yield 82%): **1H NMR (400 MHz, $CDCl_3$): 4.56 (m, 1H), 3.85 (m, 1H), 3.71 (m, 1H), 3.65 (m, 1H), 3.61 (m, 1H), 3.48 (m, 1H), 3.36 (m, 1H), 1.51 (m, 6H), 1.42 (s, 9H), 1.35-1.23 (m, 26H), 1.1 (d, 3H)**

The following synthesis route was performed according to ref. (4):

Preparation of (7):

180 mg (6) were dissolved in 2.5 ml DCM. After addition of 0.25 ml pyridine, 0.125 ml acetic anhydride and 20 mg DMAP, the mixture was stirred for 1 h at RT. 2.5 ml DCM, 20 ml hexane/EtOAc (1:1) and 20

ml brine were added. The organic phase was separated and dried. 154 mg (7) (yield 78%) was recovered by silica gel chromatography (hexane:EtOAc, 3:1).

Preparation of (8):

150 mg (7) was dissolved in 3 ml acetone and 1 ml MeOH. 15 mg toluene sulfonic acid was added and the reaction stirred for 4 ½ h at RT. 3 ml acetone, 1 ml MeOH, 20 ml hexane/EtOAc (1:1) and 10 ml sat. aq. $NaHCO_3$ were added. The organic layer was separated, dried and the residue purified by silica gel chromatography (hexane:EtOAc, 2:1) to give 65 mg (8) (yield 50%): **1H NMR (400 MHz, $CDCl_3$): 4.83 (m, 1H), 4.6 (d, 1H) , 3.6 (t, 2H), 2.04 (s, 3H), 1.53 (m, 4H), 1.41 (s, 9H), 1.35-1.22 (m, 22H), 1.06 (d, 3H)**

Preparation of (10):

65 mg (8) were dissolved in 1.7 ml DCM. After addition of 70 mg powdered molecular sieves and 75 mg pyridiniumchlorochromate (PCC), the mixture was stirred for 1 h at RT. The solvent was evaporated and the residue extracted with 2x 5 ml hexane/EtOAc (3:1) and purified by silica gel chromatography to yield 50 mg (9) (yield 75%)

50 mg (9) were dissolved in 2 ml MeOH and stirred with 70 mg potassium carbonate and 70 µl Bestmann reagent (Dimethyl-(1-diazo-2-oxopropyl)phosphonate) overnight at RT and subsequently for 1 h at 45 ºC. After addition of 60 ml hexane/EtOAc (1:1) and 20 ml brine, the organic layer was separated and dried. The product was purified by silica gel chromatography (hexane:EtOAc, 3:1) to yield 50 mg (10): **1H NMR (400 MHz, $CDCl_3$): 4.74 (d, 1H), 3.61 (m, 1H), 3.56 (m, 1H), 2.1 (td, J=7.06 Hz, 2.65 Hz, 2H), 1.86 (t, J= 2.65 Hz, 1H), 1.45 (m, 4H), 1.38 (s, 9H), 1.35-1.17 (m, 22H), 1.0 (d, 3H)**

Preparation of (11):

50 mg (10) were dissolved in 4 ml THF and 1 ml conc. HCl and stirred for 3 h at RT. The solvent was evaporated and the residue dissolved in 20 ml water. The water phase was extracted with 20 ml hexane, the water phase was adjusted to approx. pH 10 by NaOH addition and the product was extracted with 6x 10 ml DCM. The pooled DCM phase was evaporated and the residue purified by silica gel chromatography ($CHCl_3$:MeOH:NH_3, 40:10:1) to give 20 mg (11): **1H NMR (400 MHz, CD_3OD: $CDCl_3$, 2:1): 3.47 (m, 1H), 2.88 (m, 1H), 2.13 (td, J= 6.92 Hz, 2.65 Hz, 2H), 2.03 (t, J=2.65Hz, 1H), 1.48 (m, 4H), 1.31-1.2 (m, 20H), 1.05 (d, 3H)**

13C NMR (400 MHz, CD_3OD: $CDCl_3$, 2:1): 85.14, 74.87, 68.99, 51.82, 33.65, 30.53, 30.47, 30.44, 30.36, 29.95, 29.55, 29.40, 27.00, 19.00, 15.80

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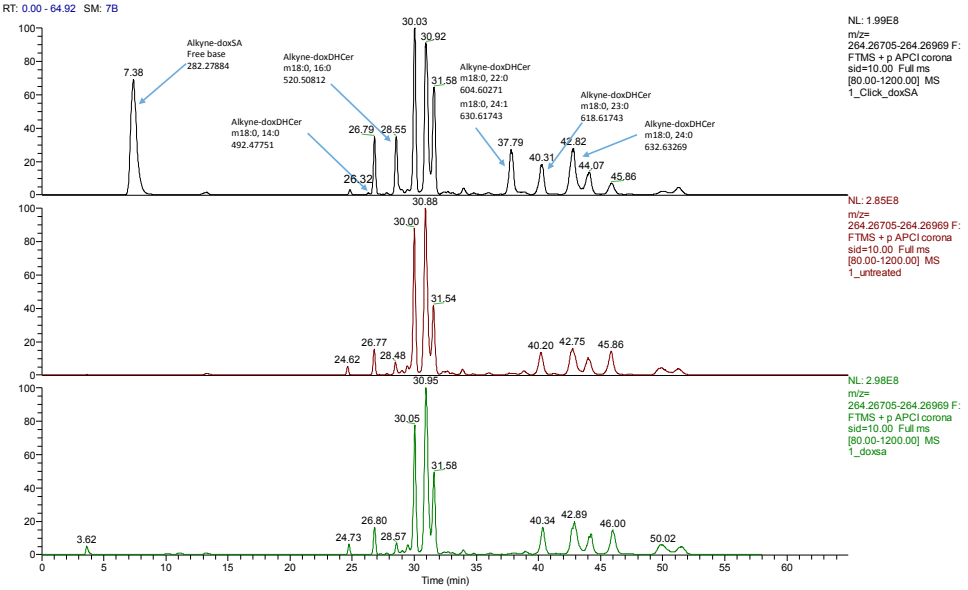
Identification of alkyne-sphingolipid species by MS

A



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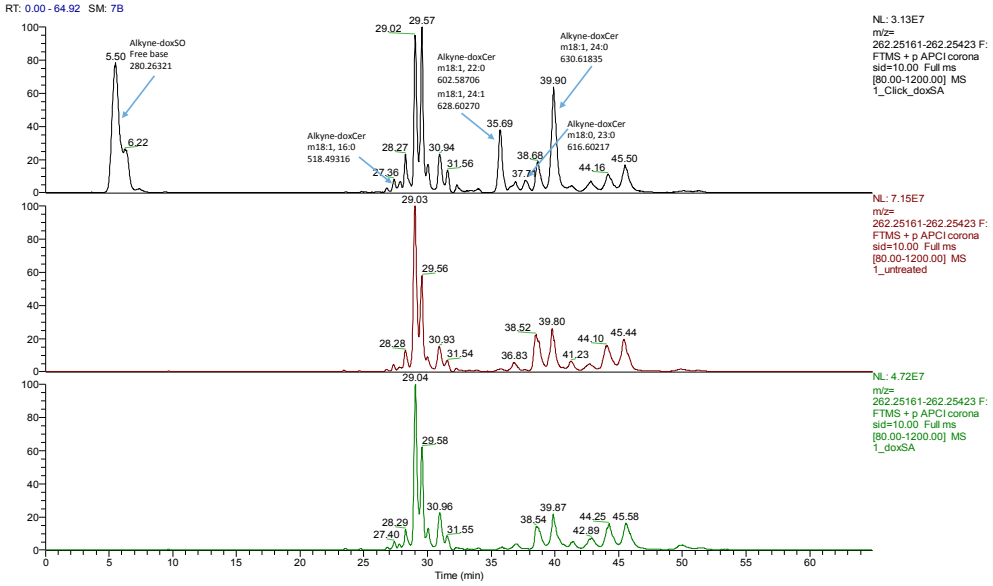


Supplemental Figure S3. Representative selected ion chromatograms used for the identification of alkyne-doxDHCer (A) and alkyne-doxCer (B) species

Identification of alkyne-doxDHCer (A) and alkyne-doxCer (B) species was based on a comparison with untreated and unlabeled doxSA-treated cells. The mass of the non-fragmented molecular ions for all of the peaks which appeared only in the alkyne-doxSA treated cells were then checked, as well as the loss of only one water molecule in the source and not two, which would indicate a ceramide. The relative retention times of the identified species were also analyzed, which allowed for confirmation of the species indicated on the figure.

Supplemental Table S1. Identification of alkyne- and deuterated Cer, doxDHCer, and doxCer species by LC-MS

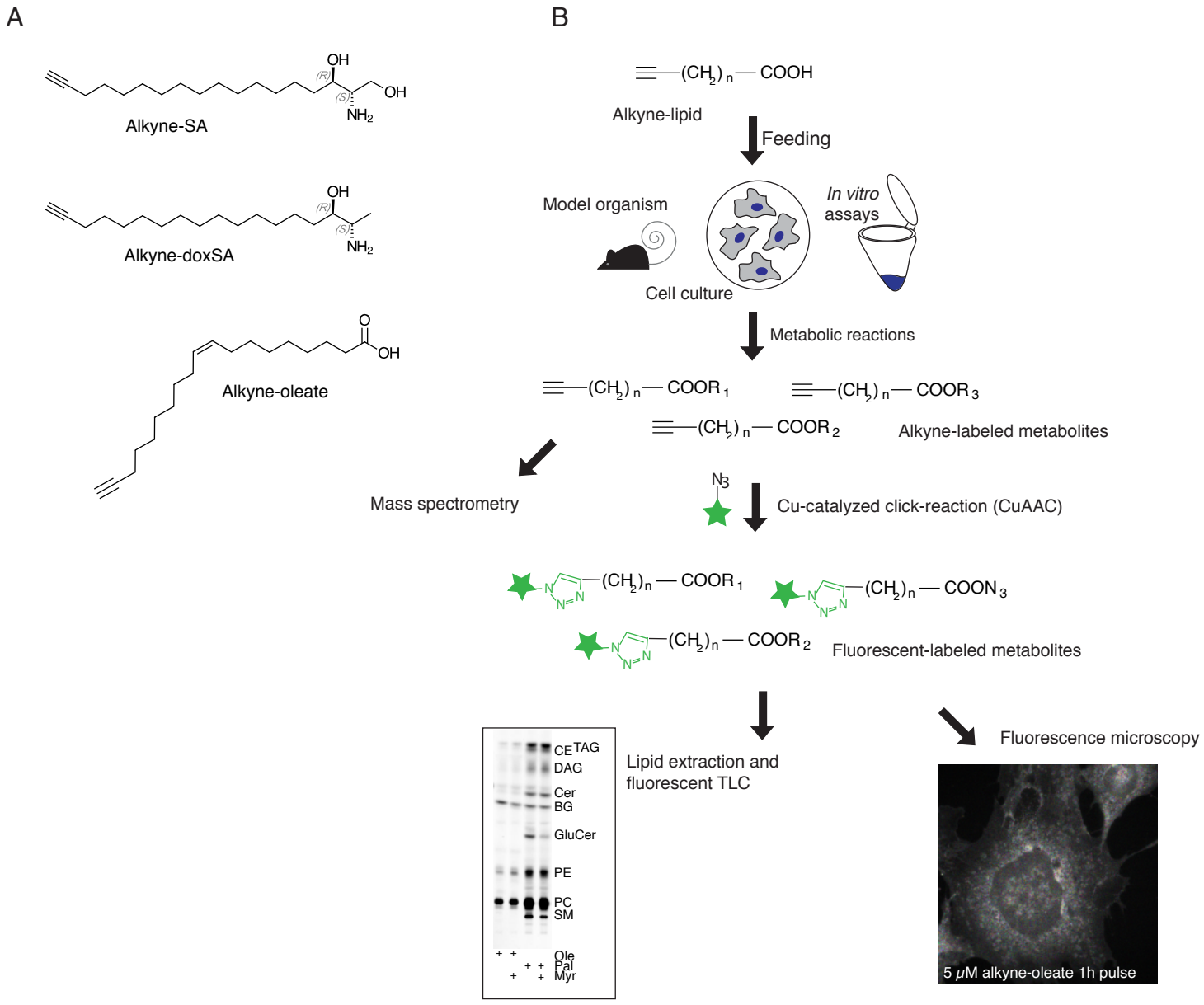
Alkyne-doxDHCer	Formula	Full mass	-H2O	Other identifying fragment	Retention time
m18:0,14:0	C32H61O2N	492.47751	474.46749	264.26837	26.3
m18:0,16:0	C34H65O2N	520.50881	502.49879	264.26837	28.6
m18:0,20:0	C38H73O2N	576.57141	558.56139	264.26837	33.9
m18:0,22:0	C40H77O2N	604.60271	586.59269	264.26837	37.8
m18:0,23:0	C41H79O2N	618.61836	600.60834	264.26837	40.3
m18:0,24:0	C42H81O2N	632.63401	614.62399	264.26837	42.8
m18:0,24:1	C42H79O2N	630.61836	612.60834	264.26837	37.8

Alkyne-doxCer	Formula	Full mass	-H2O	Other identifying fragment	Retention time
m18:1,16:0	C34H63O2N	518.49316	500.48314	262.25256	27.4
m18:1,22:0	C40H75O2N	602.58706	584.57704	262.25256	35.7
m18:1,23:0	C41H77O2N	616.60271	598.59269	262.25256	37.7
m18:1,24:0	C42H79O2N	630.61836	612.60834	262.25256	39.9
m18:1,24:1	C42H77O2N	628.60271	610.59269	262.25256	35.7

Alkyne-Cer	Formula	Full mass	-H2O	-2H2O	Other identifying fragment	Retention time
d18:1, 16:0	C34H63O3N	516.4780551	498.4680388	480.4580225	262.25284	30.4
d18:1, 24:0	C42H79O3N	628.6032556	610.5932393	592.583223	262.25284	40.9
d18:1, 24:1	C42H77O3N	626.5876055	608.5775892	590.5675729	262.25284	37.4

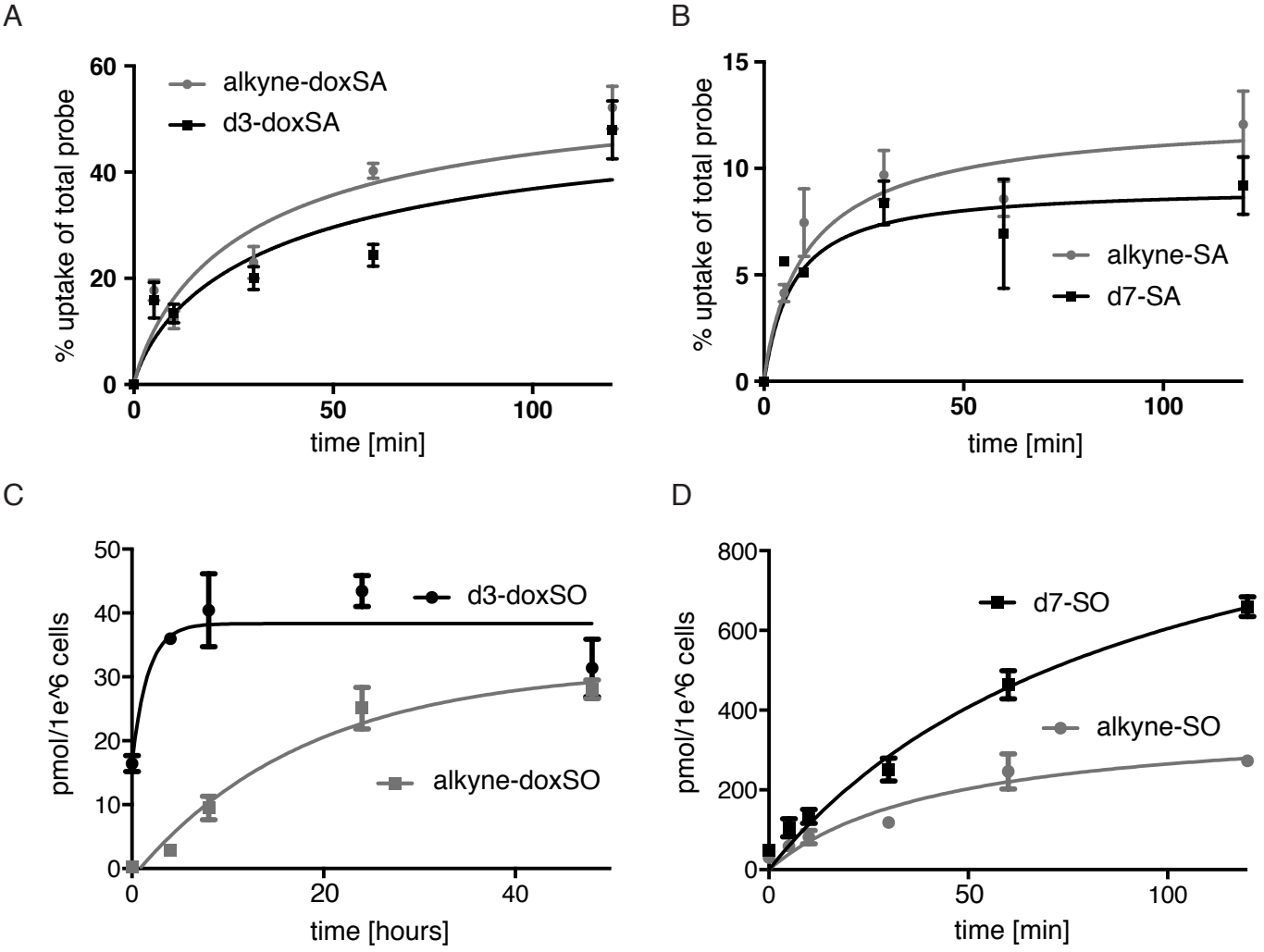
D3-doxDHCer	Formula	Full mass	-H2O	Other identifying fragment	Retention time
m18:0,14:0	C32H62[2]H3O2N	499.52764	481.51762	271.31871	31.4
m18:0,16:0	C34H66[2]H3O2N	527.55894	509.54892	271.31871	34.5
m18:0,20:0	C38H74[2]H3O2N	583.62154	565.61152	271.31871	44.3
m18:0,22:0	C40H78[2]H3O2N	611.65284	593.64282	271.31871	50.7
m18:0,23:0	C41H80[2]H3O2N	625.66849	607.65847	271.31871	55.2
m18:0,24:0	C42H82[2]H3O2N	639.68414	621.67412	271.31871	61.2
m18:0,24:1	C42H80[2]H3O2N	637.66849	619.65847	271.31871	50.6

D3-doxCer	Formula	Full mass	-H2O	Other identifying fragment	Retention time
m18:1,16:0	C34H64[2]H3O2N	525.54329	507.53327	269.30306	31.9
m18:1,22:0	C40H76[2]H3O2N	609.63719	591.62717	269.30306	45.3
m18:1,23:0	C41H78[2]H3O2N	623.65284	605.64282	269.30306	48.7
m18:1,24:0	C42H80[2]H3O2N	637.66849	619.65847	269.30306	53.1
m18:1,24:1	C42H78[2]H3O2N	635.65284	617.64282	269.30306	45.2



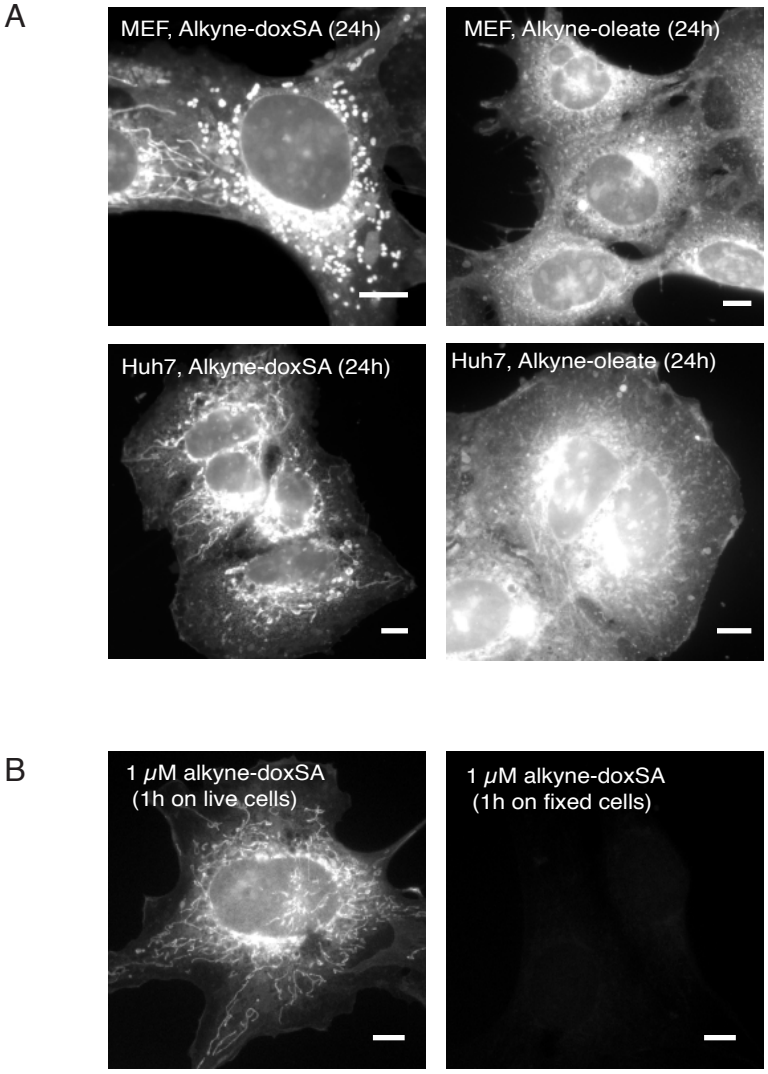
Supplemental Figure S4. Alkyne lipids (A) and click methods used in this study (B)

Alkyne lipids can be used in *in vitro*, *in situ* or *in vivo* experiments. Subsequent to their metabolic conversion, they are detected by mass spectrometry or via covalent linkage of the alkyne group to an azido-reporter molecule by the highly specific CuAAC- click reaction. By clicking a fluorescent azido reporter molecule to the alkyne group, the lipid metabolism of the alkyne probe can be visualized via a fluorescent TLC, or the subcellular localization of an alkyne lipid can be detected by fluorescence microscopy in fixed cells. TAG: triacylglycerol; CE: cholesterolester; DAG: diacylglycerol; Cer: ceramide; BG: background; GluCer: glucosylceramide; PE: phosphatidylethanolamine; PC: phosphatidylcholine; SM: sphingomyelin; Ole: oleate; Pal: palmitate; Myr: myriocin (Inhibitor for serine palimtoyltransferase)



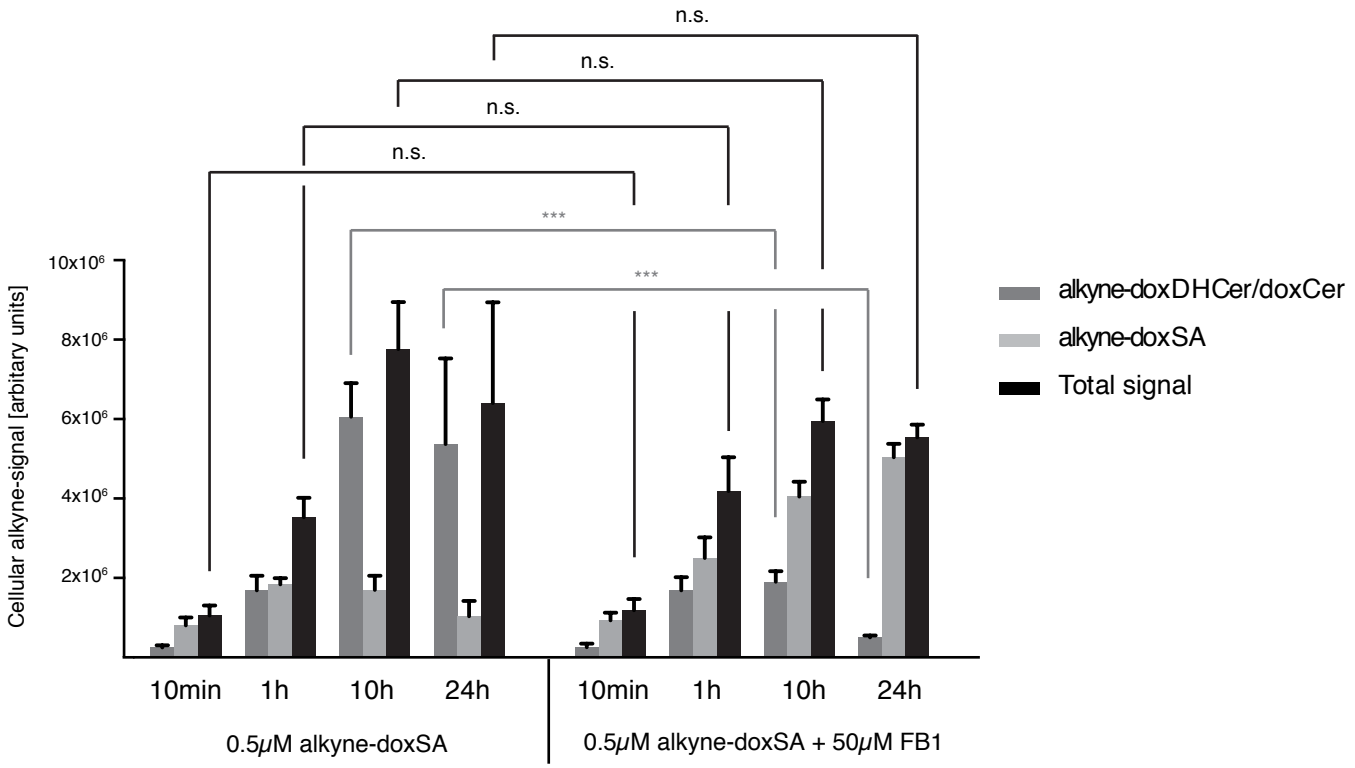
Supplemental Figure S5. Uptake (A,B) and desaturation (C,D) of alkyne and deuterated sphingoid probes

Cellular uptake of alkyne-doxSA, d3-doxSA **(A)** and alkyne-SA, d7-SA **(B)** was analyzed by treating MEF cells with 1 μM of the lipid probes and measuring the total labeled cellular sphingoid base content as compared to what was left in the medium at 0, 5, 10, 30 min, 1 hour, 2 hours. The data is shown as a % of the total labeled lipid measured in the cells + medium. To study downstream metabolism of the labeled sphingoid base probes, the appearance of total labeled alkyne-doxSO and d3-doxSO **(C)** or alkyne-SO and d7-SO **(D)** was analyzed by acid/base hydrolysis and LC-MS. Please note the difference in the x-axis time scale between **C** and **D**. Data are presented as average ± s.d.



Supplemental Figure S6. Alkyne-doxSA treatment compared to alkyne-oleate treatment (A) and alkyne-doxSA treatment of live compared to fixed cells (B)

A. MEF or HuH7 cells were treated for 24 hours with either 1 μM alkyne-doxSA or 1 μM alkyne-oleate. Cells were then fixed and the alkyne moiety reacted with ASTM-BODIPY. Scale bars represent 10 μm. **B**. MEF cells were treated for 1 hour with 1 μM alkyne-doxSA in growth medium before or after fixation.The alkyne label was detected with ASTM-BODIPY. Scale bars represent 10 μm.



Supplemental Figure S7. Cellular uptake of alkyne-doxSA is independent of ceramide synthase activity

MEF cells were treated with alkyne-doxSA and the ceramide synthase inhibitor (FB1) for the indicated times. Uptake of the alkyne-probe was quantified by click-fluorescent TLC. Please note the significantly reduced levels of alkyne-doxCer after 10 and 24 hours FB1 treatment, while total levels af alkyne-signal (sum of alkyne-doxSA and alkyne-doxCer) were unchanged. Data are presented as average ± s.d. (* < 0.05, **< 0.01, ***<0.001).