# Alkyne lipids as substrates for click-chemistry based in vitro enzymatic assays

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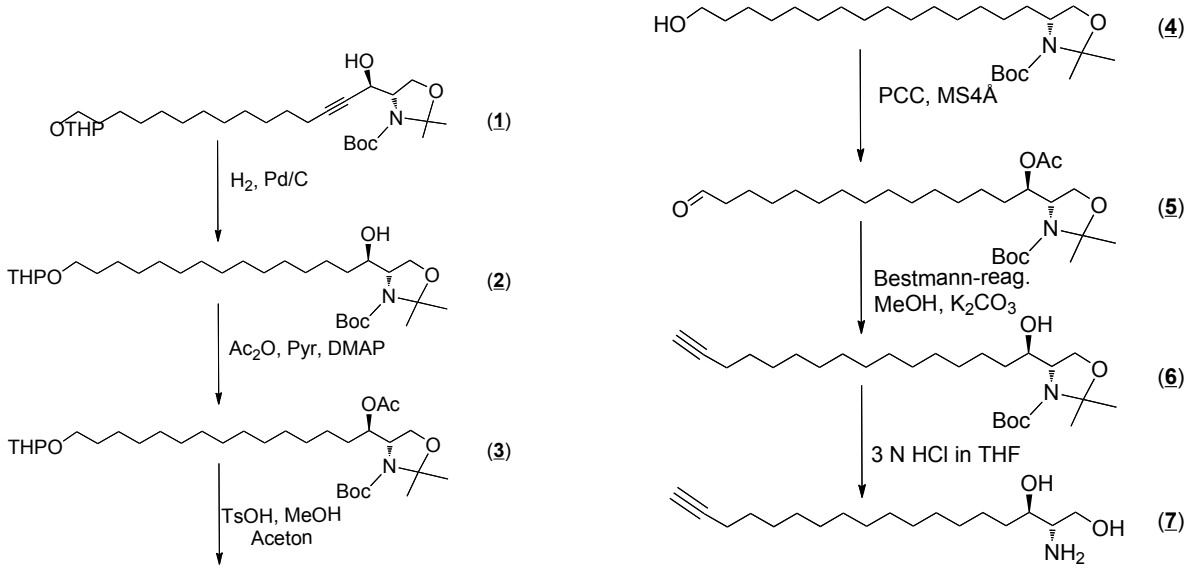
## Supplementary information

### Synthetic procedures

***Alkyne-sphinganine ((2S,3R)-2-aminooctadec-17-yn-1,3-diol*)**




*tert*-Butyl-(4*S*,1'*R*)-4-(1'-Hydroxypentadec-2'-yn-15-(2-tetrahydropyranyloxy)-yl-2,2-dimethyl-3-oxazolinecarboxylate **(1)** was synthesized from THP-protected 1-tetradecyn-14-ol and Garners aldehyde according to (1).

*tert*-Butyl-(4*S*,1'*R*)-4-(1'-Hydroxypentadec-15-(2-tetrahydropyranyloxy)-yl-2,2-dimethyl-3-oxazolinecarboxylate **(2)**
400 mg of **(1)** were dissolved in 10 ml EtOH. After addition of 40 mg 10% Pd/C, the mixture was stirred for 2 h at 1 bar $H_2$ at RT. 40 ml EtOH were added and the catalyst pelleted by centrifugation at 5000 *g* for 15 min. Evaporation of the solvent yielded the product in quantitative yield.

1H-NMR (400 MHz, $CDCl_3$) 4.55 (m, 1H, THP C2*H*), 4.1-3.3 (5 x m, 8H, C4*H,* C5$H_2$, C1'*H,* C15'$H_2$, THP C6$H_2$), 1.8 and 1.68 (2 x m, 2H, THP C3$H_2$), 1.6-1.36 ppm (m, b, 23H, C2'$H_2$, C14'$H_2$, THP C4$H_2$, THP C5$H_2$, 5 x methyl-C$H_3$) 1.36-1.1 (m, b 22H, C3'-13'$H_2$),

*tert*-Butyl-(4*S*,1'*R*)-4-(1'-Acetoxypentadec-15-hydroxy)-yl-2,2-dimethyl-3-oxazolinecarboxylate **(4)**

370 mg of **(2)** were dissolved in 5 ml DCM. After addition of 0.5 ml pyridine, 250 µl acetic anhydride and 40 mg DMAP, the mixture was stirred for 30 min at RT. After addition of 20 ml hexane/ethyl acetate 1/1 and 20 ml brine, the organic phase was collected and dried to give the acetylated product in high purity and yield. The residue **(3)** was dissolved in a mixture of 6 ml acetone, 2 ml MeOH and 30 mg toluenesulfonic acid. After stirring at RT for 2 h, 20 ml hexane/ethyl acetate 1/1 and 10 ml sat. aq. $NaHCO_3$ were added. The organic layer was separated, evaporated and the residue purified by silica gel chromatography (solvent gradient hexane/ethyl acetate 3/1 to 1/1) to give 230 mg pure **(4)**.

1H-NMR (400 MHz, $CDCl_3$) 5.33 (m, 1H, C1'*H*), 4.1-3.8 (3 x m, 3H, C4*H,* C5$H_2$), 3.61 (t, 2H, C15'$H_2$), 2.04 (s, 3H, acetyl C$H_3$), 1.6-1.38 ppm (m, b, 19H, C2'$H_2$, C14'$H_2$, 5 x methyl-C$H_3$) 1.38-1.1 (m,b 22H, C3'-13'$H_2$)

*tert*-Butyl-(4*S*,1'*R*)-4-(1'-Hydroxyhexadec-15-yn)-yl-2,2-dimethyl-3-oxazolinecarboxylate **(6)**

a) PCC oxidation to the aldehyde: 200 mg of **(4)** were dissolved in 5 ml DCM. After addition of 200 mg powdered molecular sieves and 220 mg pyridiniumchlorochromate, the mixture was stirred for 30 min at RT. The solvent was evaporated and the residue extracted with 3 x 10 ml hexane/ethyl acetate 3/1. The extracts were evaporated and the aldehyde **(5)** isolated by silica gel chromatography (solvent hexane/ethyl acetate 1/1).

b) Bestmann-Ohira reaction: the aldehyde was stirred with 140 mg Dimethyl (1-Diazo-2-oxopropyl)phosphonate (Bestmann-Ohira reagent) and 200 mg potassium carbonate in 6 ml MeOH for 30 min at RT. TLC control showed formation of the alkyne and a weak band of lower mobility. Upon prolonged stirring (3 h at RT, 1 h at 45 °C) more than 90% of the material was found in the lower band. After addition 20 ml hexane/ethyl acetate 1/1 and 20 ml brine, the organic phase was collected and dried and the residue purified by silica gel chromatography (solvent hexane/ethyl acetate 3/1) to give 130 mg pure **(6)**

1H-NMR (400 MHz, $CDCl_3$): 4.1-3.4 (m, 4H, C1'*H,* C4*H,* C5$H_2$), 2.16 ppm (dt, J=7.2 Hz, 2.7 Hz, 2H, C14'$H_2$), 1.92 (t, J=2.7 Hz, 1H, C16'*H*), 1.6-1.37 ppm (m, b, 19H, C2'$H_2$, C13'$H_2$, 5 x methyl-C$H_3$) 1.37-1.1 (m,b 20H, C3'-12'$H_2$)

(2*S*,3*R*)-2-Aminooctadec-17-yn-1,3-diol **(7)**

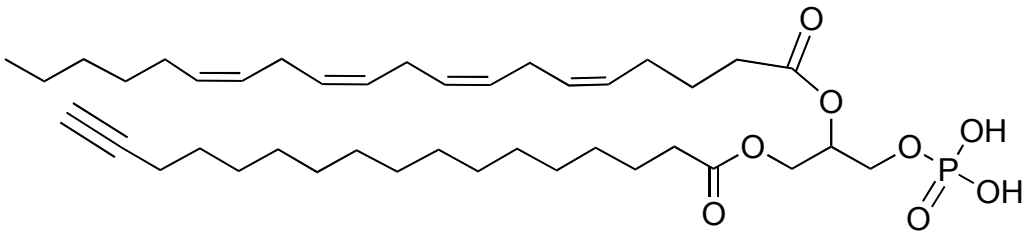
100 mg of **(6)** were dissolved in 4 ml THF and 1 ml conc. HCl and stirred for 2 h at RT. The solvent was evaporated in vacuo, the residue dissolved in 10 ml water and the water phase extracted with 10 ml hexane/diethyl ether 4/1. The aqueous phase was

brought to pH 10 by addition of NaOH and extracted with 4 x 5 ml of DCM. The pooled DCM phases were evaporated and the residue purified by silica gel chromatography (solvent $CHCl_3$/MeOH/aq. $NH_3$ 40/10/1) to give 47 mg pure **(7)**.
1H-NMR (400 MHz, $CD_3OD$): 3.72 ppm (dd, 1H, C1a*H*), 3.50 (m, J=7.2, 1H, C3*H*), 3.46 (dd, 1H, C1b*H*), 2.71 (ddd, 1H, C2*H*), 2.15 (m, 3H, C16$H_2$, C17*H*), 1.6-1.45 ppm (m, b, 4H, C4$H_2$, C15$H_2$) 1.45-1.2 (m,b 20H, C5-14$H_2$)
13C-NMR (400 MHz, $CDCl_3$+10% $CD_3OD$): 84.75 ppm (C17), 73.88 (C3), 67.97 (C18), 63.05 (C1), 55.65 (C2), C4-C16: 33.66, 29.60, 29.54 (large signal), 29.51, 29.41, 29.02, 28.67, 25.95, 18.28.

***Dihydroceramides (N-Stearoyl-D-erythro-sphinganine, 18:0-dhCer and N-Nervonoyl-D-erythro-sphinganine, 24:1-dhCer)***
The dihydroceramides were synthesized from alkyne-sphinganine and stearic acid or nervonic acid. The acid was transformed to the NHS-ester with NCS (N-chlorosuccinimide) and DCC (Dicyclohexylcarbodiimide) and alkyne-sphinganine was added to the reaction mix. The crude products were purified by preparative TLC.
ESI-MS for *N*-Stearoyl-D-erythro-sphinganine: calculated for $C_{36}H_{69}NO_3$: 564.6 $[M+H]^+$, 586.5 $[M+Na]^+$, found: 564.6 $[M+H]^+$, 586.5 $[M+Na]^+$. ESI-MS for *N*-Nervonoyl-D-erythro-sphinganine: calculated for $C_{42}H_{79}NO_3$: 668.6 $[M+Na]^+$, found: 668.6 $[M+Na]^+$.

***Alkyne-PAPA (1-(16-heptadecynoyl)-2-arachidonyl-sn-glycerol-3-phosphate) and alkyne-OLPA (1-(Nonadec-9-cis-en-18-ynoyl)-sn-glycerol-3-phosphate)***

***Alkyne-PAPA*** was prepared by phosphorylation (2) of 1-(heptadec-16-ynoyl)-2-arachidonyl-*sn*-glycerol, which was synthesized by sequential acylation of protected glycerol using the procedure described by (3) with minor modifications.




Trimethylphosphite (67 mg, 0.54 mmol) was dissolved in 1.5 ml DCM and reacted with 124 mg (0.49 mmol) iodine. The mixture was then added dropwise with stirring at 0°C to a solution of 282 mg (0.45 mmol) 1-(heptadec-16-ynoyl)-2-arachidonyl-*sn*-glycerol and 142 µl pyridine in 5 ml DCM. After 2 h, the reaction was allowed to come to RT, the solvents were evaporated in vacuo and the residue subjected to silica column chromatography (hexane/ethyl acetate 2/1 to 1/1) to obtain the protected PA. For deprotection, the material (about 300 mg) was dissolved in 1 ml DCM and treated with

300 µl bromotrimethylsilane for 2 h. After drying in vacuo, the residue was stirred with 5 ml $CHCl_3$ and 400 µl 25% $NH_3$ for 30 min. After drying in vacuo, the residue was subjected to silica column chromatography ($CHCl_3$/MeOH/5% aq. $NH_3$ 65/24/5) to obtain 240 mg of Alkyne-PAPA.

1H-NMR (400 MHz, $CDCl_3$): 5.38 ppm (m, 8 H, olefinic C*H*), 5.25 (m, 1 H, glycerol C2*H*), 4.39, 4.19, 3.95 (dd 1 H, m 1H, m 2 H, glycerol C1,3$H_2$), 3.0-2.7 (m, 6H, arach. C7,10,13C$H_2$), 2.4-2.25 (m, 4H, fatty acid C2$H_2$), 2.19 (dt, 2H, alkyne-Pal C15$H_2$), 2.15-2.0 (m,b 4H, arach. C4,16$H_2$) C19*H*), 1.95 (t, 1H, alkyne-Pal C17*H*), 1.75-1.5 (m, 6H, fatty acid C3$H_2$, alkyne-Pal C14$H_2$), 1.5-1.2 (m,b 26H, alkyne-Pal C4-13, arach. C17-19 C$H_2$), 0.91 (t, 3H, arach. C20$H_3$)

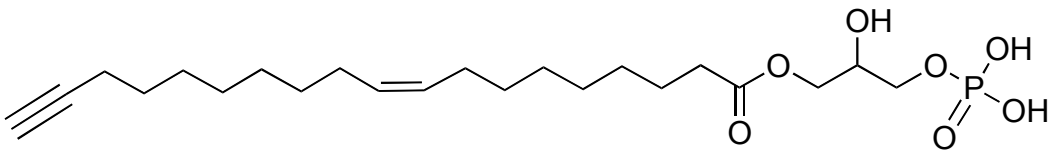
***Alkyne-OLPA*** was synthesized by selective acylation of *sn*-glycerol-3-(diethylphosphate) and subsequent deprotection:

***sn-glycerol-3-(diethylphosphate)***

A mixture of 1.24 g (4.9 mmol) iodine and 900 mg (5.4 mmol) triethylphosphite in 15 ml DCM was slowly added to a solution of *D*-isopropylideneglycerol (*sn*-3-OH; 594 mg, 4.5 mmol) and 1.42 ml pyridine in 25 ml DCM. After stirring for 1 h, the pyridinium salt was pelleted by centrifugation and the solvent was evaporated in vacuo. The residue was subjected to silica column chromatography (hexane/ethyl acetate/ethanol 50/50/3). 1.1 g of the protected intermediate were dissolved in 100 ml MeOH followed by addition of 100 µl acetyl chloride. The mixture was stirred for 16 h, 100 µl triethylamine were added and the solvent evaporated in vacuo. The residue was dissolved in 10 ml ethyl acetate, centrifuged and the supernatant evaporated to obtain 900 mg product.

1H-NMR (400 MHz, $CDCl_3$/$CD_3OD$ 2/1) 4.43 (s, 2H, -O*H by exchange with MeOD*), 4.15 (m, 4H, ethyl-C$H_2$), 4.0-3.9 (m, 2H, C2*H* + C1*H*), 3.77 (m, 1H, acetyl C1*H*), 3.52 (m, 2H, C3$H_2$, 1.28 (2 x t, 6H, ethyl-C$H_3$)

***Alkyne-OLPA***




Alkyne-oleic acid (150 mg, 0.5 mmol) was stirred with 2 ml thionylchloride at 65°C for 2 h. Excess thionylchloride was evaporated in vacuo and the residue (i. e. the acyl chloride) dissolved in 2 ml DCM. This solution was slowly added at -78°C to a stirred solution of 145 mg (0.6 mmol) *sn*-glycerol-3-(diethylphosphate) and 140 µl (1.2 mmol) 2,6-lutidine in 3 ml DCM. After stirring for 2 h at -78°C and 30 min at RT, 100 µl MeOH were added and the solvents evaporated in vacuo. The residue was subjected to silica column chromatography (hexane/ethyl acetate/MeOH 50/50/3)) to obtain 149 mg of

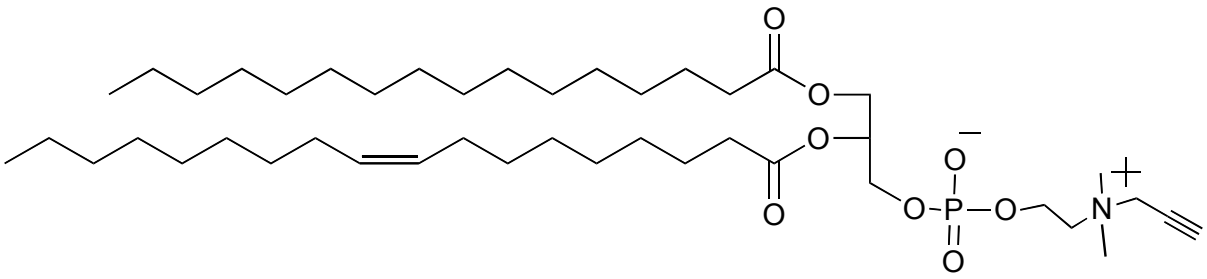
the protected alkyne-OLPA. For removal of the ethyl groups, 120 mg were treated with 500 µl DCM, 200 µl *N*,*O*-bis-trimethylsilylacetamide and 400 µl bromotrimethylsilane for 1 h. After removal of all solvent, the residue was dissolved in 5 ml 5% water in MeOH and stirred for 30 min. 100 ml 25% aq. $NH_3$ were added, the solvents removed in vacuo, and the residue subjected to silica column chromatography ($CHCl_3$/MeOH/$H_2O$ 40/50/10) to obtain 50 mg of alkyne-OLPA.
1H-NMR (400 MHz, $CDCl_3$/$CD_3OD$ 2/1): 5.32 ppm (m, 2H, olefinic C*H*), 4.64 (s, broad 6H, -O*H and* N*H3 by exchange with MeOD*), 4.2-3.7 (5 x m, 5H, glycerol C*H*), 2.33 (t, 2H, C2$H_2$), 2.18 ppm (dt, 2H, C17$H_2$), 2.08-1.95 (m,b 5H, C8,11$H_2$, C19*H*), 1.62 (m, 2H, C3$H_2$), 1.52 (m, 2H, C16$H_2$), 1.43-1.25 (m,b 16H, C4-7,12-15$H_2$)

***Alkyne-oleoyl-CoA (nonadec-9-cis-en-18-ynoyl coenzyme A)***

The synthetic procedure was adapted from (4), purification from (5).
Alkyne-oleate (3 mg) was dissolved in 100 µl dry THF and a solution of carbonyldiimidazole (1.8 mg) in 100 µl THF was added. After incubation at RT for 30 min, the solvent was evaporated and the residue dissolved in 200 µl THF/$H_2O$ 2/1 After the addition of CoASH (8.2 mg) in 0.5 ml THF/$H_2O$ 2/1, the pH of the reaction mix was adjusted to 7.0-7.5 with 1 N NaOH and the reaction was incubated under Ar for 4 h. THF was evaporated and MOPS buffer (pH 7.4) added to a concentration of 100 mM. The reaction mix was loaded directly on a Sep-Pak C18 column, which had been activated with MeOH and equilibrated with MOPS buffer. After application of the reaction mix, the column was washed/ eluted with 0.5 ml of 100 mM MOPS pH 7.4, 1 ml MeOH/$H_2O$ 1/1 and 10 ml MeOH, consecutively. Fractions were analyzed by TLC ($H_2O$/n-butanol/acetic acid 30/50/20), and the product containing fractions combined and evaporated. Residual alkyne-oleate was removed by extraction: To the methanolic crude product solution the same volume of an aqueous solution of HCl (pH 3.5) was added and the aqueous phase extracted three times with 200 µl hexane. Removal of alkyne-oleate was monitored by TLC. The aqueous phase was evaporated to yield alkyne-oleoylCoA (0.6 mg). Yield and purity of the product were determined by click reaction and TLC.

***Propargyl-PC (pPC) and palmitoyl-lyso-propargyl-PC (PLpPC)***

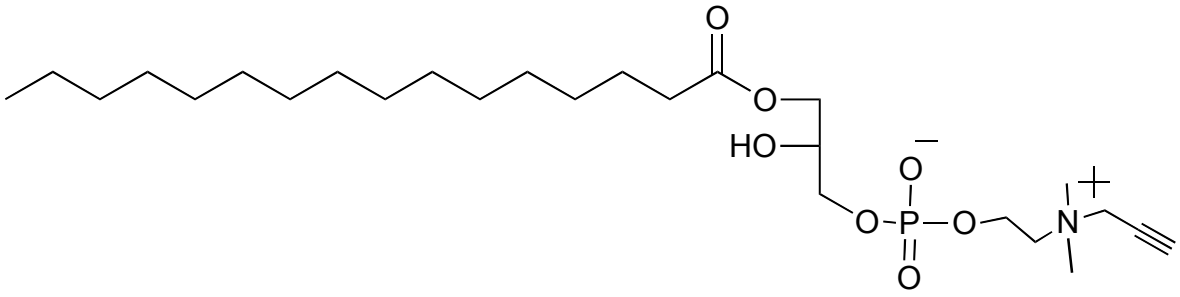
***pPC*** was synthesized by transphosphatidylation of egg yolk phosphatidylcholine:



A mixture of 6.72 g of propargylcholine bromide and 168 U [µMol/h] PLD (*Streptomyces spec*.) in 170 ml of acetate buffer (100 mM sodium acetate, pH 5.6, 40 mM $CaCl_2$) was added to a solution of 4.8 g egg yolk PC in 1.3 l diethylether. After vigorous stirring for 24 h, the organic phase was separated and the solvent evaporated. The residue was separated by silica column chromatography ($CHCl_3$/MeOH/$H_2O$ 65/25/2) to yield 3.2 g product.

1H-NMR (400 MHz, $CDCl_3$): 5.32 ppm (m, 4 H, fatty acid olefinic *H*), 5.19 (m, 1 H, C2*H*), 4.70 (d, 2 H, propargyl-C$H_2$) 4.38 (m, 2 H, POC$H_2$), 4.35 (m, 1 H, C1*H*), 4.11 (m, 1H, C1*H*), 3.98 (m, 4 H, C3$H_2$ and NC$H_2$), 3.43 (s, 6 H, N(C$H_3$)$_2$), 2.92 (t, 1H, propargyl-CC*H*), 2.27 (m, 4 H, fatty acid C2$H_2$), 2.00 (m, 4 H, fatty acid allylic C$H_2$), 1.55 (m, 4 H, fatty acid C3$H_2$), 1.17-1.30 (m, 42 H, fatty acid C$H_2$), 0.85 (t, 6 H, fatty acid C$H_3$)

***PLpPC*** by $PLA_2$ cleavage of phosphatidyl-propargylcholine

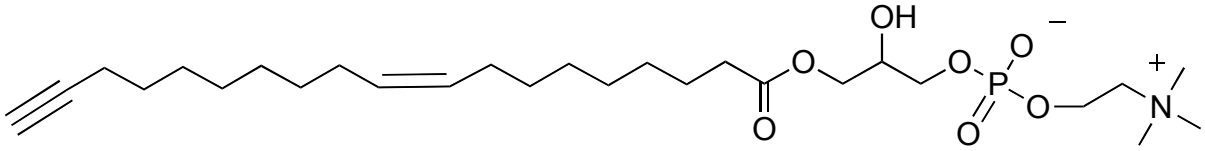


*Crotalus atrox* snake venom (5 mg) was dissolved in 5 ml buffer (0.1 M Tris-HCl, pH 8.0, 20 mM $CaCl_2$) and stirred with a solution of 1.0 g phosphatidyl-propargylcholine in 40 ml ether for 2 h at RT. The solvent was evaporated and the residue subjected to silica gel chromatography using $CHCl_3$/MeOH/$H_2O$ 65/35/8 as a solvent to yield 0.73 g product.

1H-NMR (400 MHz, $D_2O$): 4.35 ppm (d, 2 H, propargyl-C$H_2$) 4.29 (m, 2 H, POC$H_2$), 4.14 (m, 1 H, C1*H*), 4.05 (m, 1H, C1*H*), 3.97 (m, 2 H, C2$H_2$) 3.87 (m, 1 H, C3*H*), 3.75 (m, 2H, NC$H_2$), 3.65 (t, 1H, propargyl-CC*H*),) 3.25 (s, 6 H, N(C$H_3$)$_2$), 2.32 (m, 2 H, fatty acid C2$H_2$),

1.55 (m, 2H, fatty acid C3$H_2$), 1.17-1.30 (m, 25 H, fatty acid C$H_2$), 0.82 (t, 3 H, fatty acid C$H_3$)

***Alkyne-OLPC (1-(Nonadec-9-cis-en-18-ynoyl)-sn-glycerol-3-phosphocholine)***




Di-(alkyne-oleoyl)-PC (alkyne-OOPC) was prepared by acylation of *sn*3-glycerophosphocholine with alkyne-oleate:
Alkyne-oleate (200 mg) and carbonyldiimidazole (133 mg) were dissolved in 0.5 ml THF. 120 mg of a $CdCl_2$ adduct of *sn*3-glycerophosphocholine and 6.69 mg DMAP in 1 ml DMF were added. The reaction mixture was incubated in an ultrasound bath for of 4.5 h, then at 40°C for approximately 48 h. To the reaction mixture 8 ml $CHCl_3$ and 3 ml MeOH were added. The solution was washed with $H_2O$ and with MeOH/$H_2O$ 1/2. The crude product was purified over a mixed bed ion exchange column (Amberlite® MB-150 Resin, Supelco) with $CHCl_3$/MeOH/$H_2O$ 5/4/1, and by chromatography on silica gel with $CHCl_3$/MeOH/$H_2O$ (65/25/4) to yield alkyne-OOPC (46 mg).

Alkyne-OLPC: $PLA_2$ cleavage of alkyne-OOPC was performed analogously to the protocol for pPC cleavage to PLpPC.

***Alkyne-palmitoylethanolamide (N-(16-Heptadecynoyl)ethanolamine) and Alkyne-oleoylethanolamide (N-( Nonadec-9-cis-en-18-ynoyl)ethanolamine)***

Alkyne-palmitate or alkyne-oleate was reacted with DCC and NHS to yield the NHS-ester, to which 2-aminoethanol was added without prior isolation of the ester. The crude products were purified by silica gel chromatography.
1H-NMR of alkyne-palmitoylethanolamide (400 MHz, $CDCl_3$): 5.91 (b, 1H, N*H*), 3.70 (m, 2H, C1'$H_2$), 3.41 (m, 2H, C2'$H_2$), 2.7-2.5 (b, 1H, O*H*), 2.16 (2x t, 4H, C2$H_2$ and C15$H_2$), 1.92 (t, 1H, C17*H*), 1.61 (m, 2H, C3$H_2$), 1.50 (m, 2H, C14$H_2$), 1.4-1.2 (m, 20H, C4-13$H_2$)
1H-NMR of alkyne-oleoylethanolamide (400 MHz, $CDCl_3$): 6.00 (b, 1H, N*H*), 5.32 (m, 2H, C9*H* and 10*H*), 3.70 (m, 2H, C1'$H_2$), 3.40 (m, 2H, C2'$H_2$), 2.8-2.6 (b, 1H, O*H*), 2.16 (m, 4H, C2$H_2$ and C17$H_2$), 1.99 (t, 4H, C8$H_2$ and C11$H_2$), 1.92 (t, 1H, C19*H*), 1.61 (m, 2H, C3$H_2$), 1.50 (m, 2H, C16$H_2$), 1.4-1.2 (m, 16H, C4-7$H_2$, C12-15$H_2$)

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