Chapter 7

Cyclopropene probes for live-cell imaging of signaling lipids*

Introduction

Lipidomics is a relatively new discipline that studies cellular lipids on a large scale, which uses analytical chemistry principles and technological tools, particularly mass spectrometry.[1] Recent advances in mass spectrometry techniques have made it possible to detect and quantify many different lipid species in complex biological samples.[1,2] However, this approach generally requires sample homogenization followed by lipid extraction, and while bulk analysis gives valuable information on lipid biology, it does not allow for investigation of cellular localization. This spatial information is especially important in the case of lipid signaling molecules, which can be short-lived and have very localized functions.[3] Currently, detailed investigation of lipid signaling molecules is restricted by the availability of tools.

*The data presented in this chapter was gathered in collaboration with Iakovia Ttofi, Dennis Strobbe, Alexi Sarris, Herman S. Overkleeft, Sander van Kasteren, Mario van der Stelt.

Some of the most well-studied lipid signaling molecules are the endocannabinoids anandamide (AEA) and 2-arachidonoyl glycerol (2-AG), which are derivatives of the polyunsaturated arachidonic acid (AA, 20:4 n-6, **1**) (Figure 1).[4] These endocannabinoids act as lipid messengers on the cannabinoid receptors to modulate various signal transduction pathways in the brain and immune system.[5] However, even for these well-investigated lipid signaling molecules, the mechanism of their biosynthesis, intercellular transport and cellular uptake is still unclear. The most widely accepted model states that AEA and 2-AG are not pre-synthesized and stored in vesicles, but produced "on demand" and excreted through an unknown mechanism.[6] The following step, cellular reuptake of endocannabinoids, is a highly debated topic,[7–11] and different models have been proposed.[10] Research on endocannabinoid signaling would benefit from tools to visualize localization and transport of these signaling lipids *in vivo* in real-time.

A common approach for the visualization of lipids is to append a fluorescent group like nitrobenzoxadiazole.[12] However, the fluorophore affects the properties of the lipid, which may alter its localization and signaling functions.[13–15] Therefore, recent efforts have focused on introduction of small bioorthogonal tags to allow the introduction of a visualization element when required. Azide and alkyne groups have been widely used for this purpose, as they can undergo copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC),[16] strain-promoted azide-alkyne cycloaddition (SPAAC)[17] and Staudinger ligation[18] for functionalization. However, the copper catalyst required for CuAAC is incompatible with live cells,[19] SPAAC requires large strained alkynes and the Staudinger ligation has slow reaction kinetics.[20] Therefore, these approaches are less suitable to follow fast lipid signaling events in living cells.

There is an increased interest in the inverse electron-demand Diels-Alder (IEDDA) reaction, which uses electron-rich olefins like trans-cyclooctene (TCO) and tetrazine reagents, as an alternative bioorthogonal reaction to visualize rapid cellular processes.[21,22] IEDDA chemistry has fast reaction kinetics without the need for a catalyst, as well as the ability to use fluorogenic tetrazines. The fluorophore is unquenched upon conjugation of the tetrazine with a dienophile, which allows for real-time imaging of tagged molecules (Figure 1A).[23] To avoid using the moderately large TCO group as the dienophile, cyclopropenes have been investigated as an alternative olefin.[24] The reactive, strained nature of cyclopropenes provides the ability to participate in IEDDA reactions.[25,26] A number of differently substituted cyclopropenes, such as 1,3-substituted and 3,3-substituted variants, have been characterized for use in IEDDA chemistry, but not 1,2-substituted cyclopropenes.[27,28] In this chapter, the use of 1,2-substituted cyclopropene-tagged lipids for live-cell imaging is investigated, as well as the synthesis of a cyclopropene-tagged arachidonic acid for the investigation of AEA (Figure 1B).

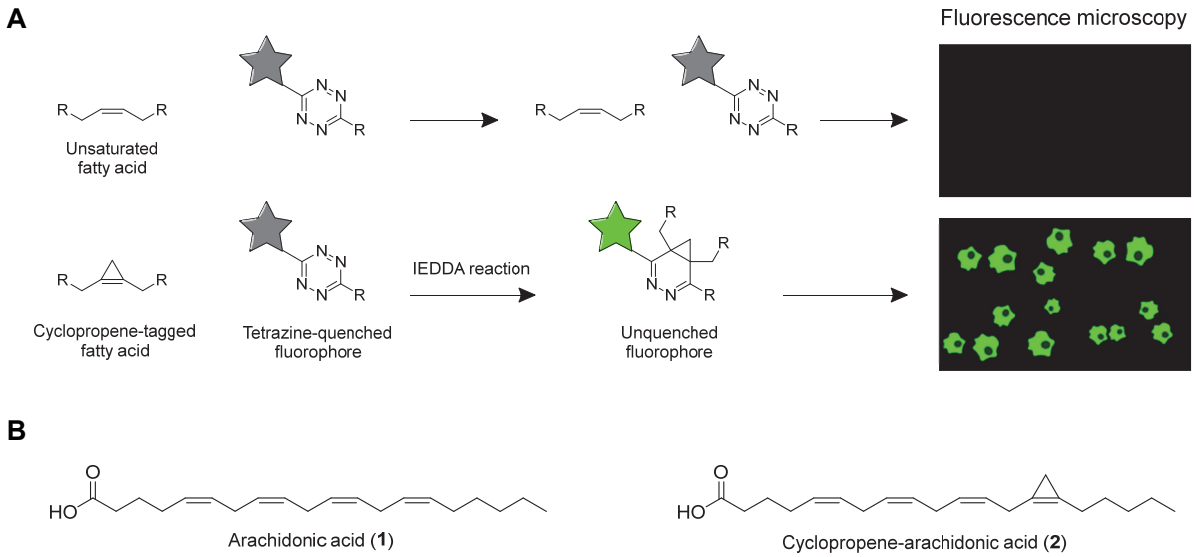


Figure 1 | Cyclopropene lipids for live-cell imaging. (A) Schematic representation of bioorthogonal labeling of cyclopropenes using tetrazine-quenched fluorophores. **(B)** Structure of arachidonic acid (**1**) and its cyclopropene analogue **2**.

Results and discussion

To investigate 1,2-substituted cyclopropene lipids in live-cell imaging using IEDDA, commercially available sterculic acid (SA, **3**) was used in tandem with oleic acid (OA, **4**) as control (Figure 2). Sterculic acid is a cyclopropene lipid naturally found in plants such as *Sterculia foetida* and was first characterized in 1952[29] and synthesized in 1960.[30] Its suitability as dienophile in IEDDA reaction with tetrazine-quenched fluorophore **5** was tested in live cells.

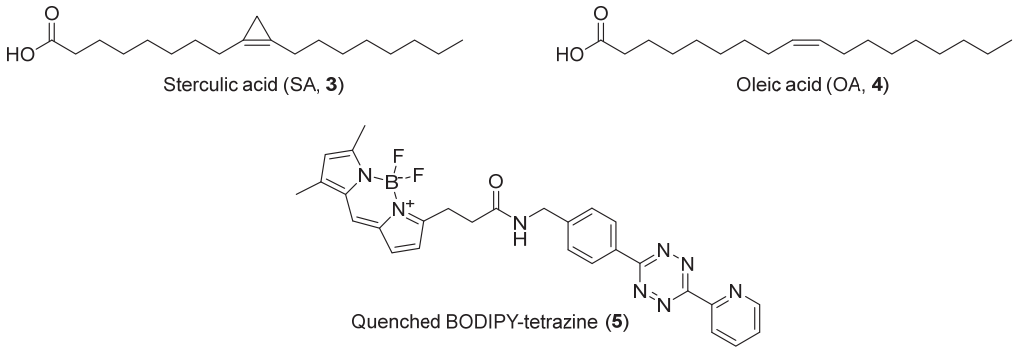


Figure 2 | Structures of oleic acid (OA, **3**), sterculic acid (SA, **4**) and BODIPY-tetrazine **5**.

U2OS cells were treated with OA (**4**) or SA (**3**) (50 µM) for 1 h, washed and subsequently incubated with different concentrations of BODIPY-tetrazine **5** for 2 h. The cells were then washed extensively and analyzed by fluorescence microscopy (Figure 3). This resulted in dose-dependent labeling of sterculic acid with the BODIPY-tetrazine, indicating successful IEDDA reaction and unquenching. Little to no fluorescence was detected in oleic acid-treated cells. The fluorescent signal seemed to be focused on the endoplasmic reticulum (ER) as opposed to the cytoplasmic membrane, which can be explained by incorporation of the free fatty acids into phospholipids through Lands' cycle and the Kennedy pathway, which occurs mainly at the ER.[31]

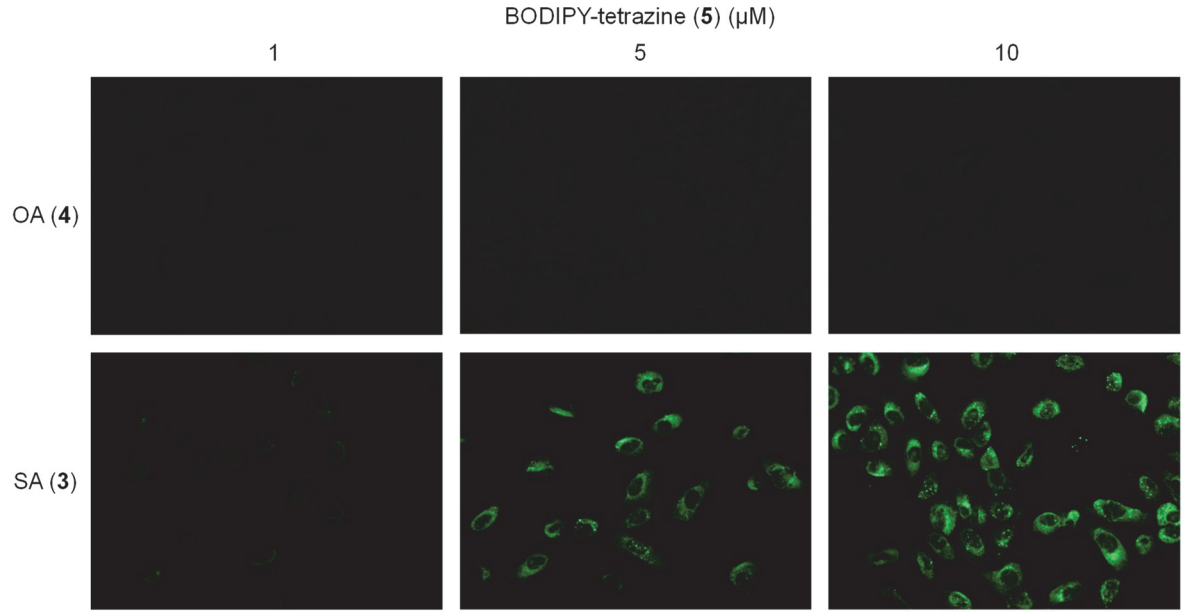


Figure 3 | Sterculic acid is dose-dependently labeled by ligation to BODIPY-tetrazine 5 in live cells. Live-cell image of U2OS cells incubated with indicated fatty acid (50 µM) for 1 h, washed and incubated with indicated concentration of BODIPY-tetrazine for 2 h, then washed and imaged.

Next, the influence of incubation time with BODIPY-tetrazine **5** was investigated to survey the progression of the reaction. U2OS cells were treated with the lipids as before, incubated for indicated time with **5**, then washed and imaged (Figure 4). This revealed that after one hour, sufficient BODIPY-tetrazine had reacted to visualize sterculic acid in the cells. However, the reaction was not complete as indicated by increased fluorescence intensity after a longer incubation time. Whether the rate-limiting step in this process is the uptake of BODIPY-tetrazine **5** or the IEDDA reaction cannot be determined with this experiment, but the reaction between **3** and **5** seems to be facilitated by accumulation of both molecules in the membrane. These results indicated that 1,2-substituted cyclopropene lipids are suitable tools to visualize uptake and localization using IEDDA chemistry.

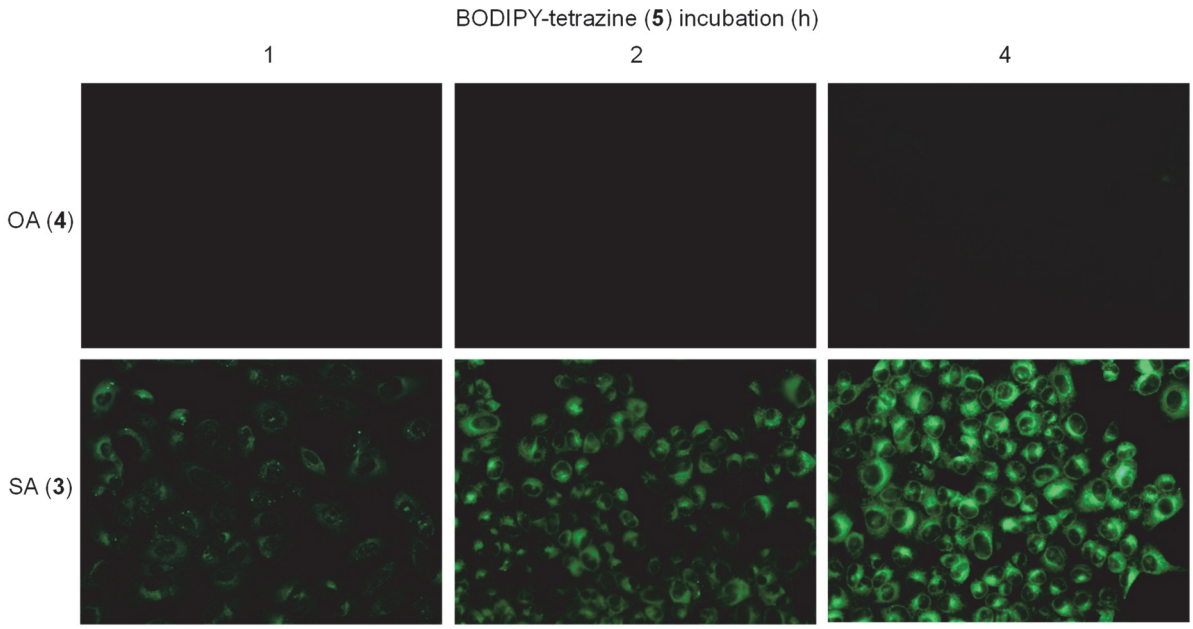
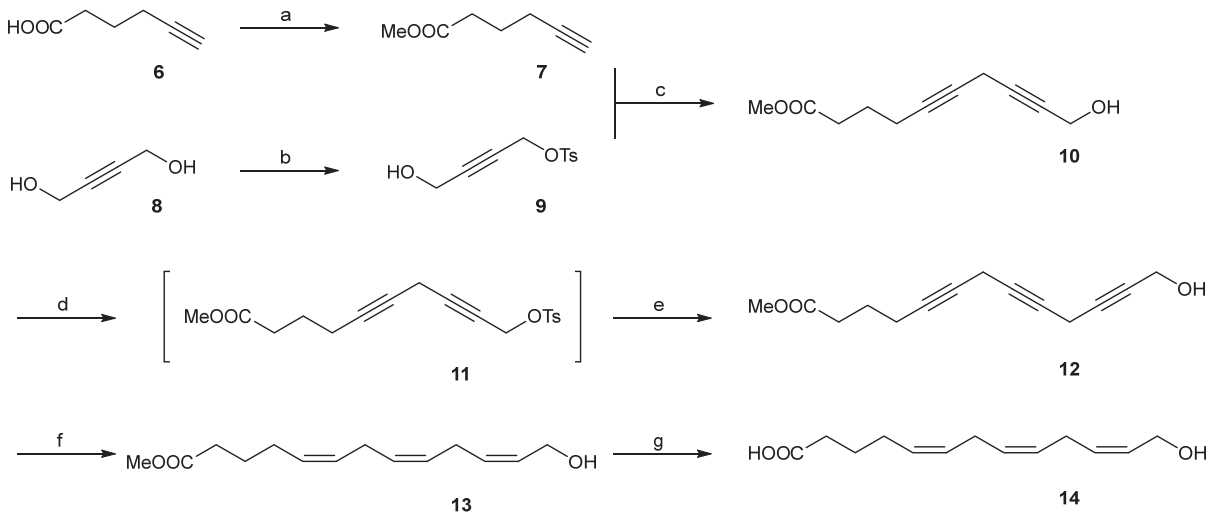


Figure 4 | Sterculic acid reacts over time with BODIPY-tetrazine in live cells. Live-cell image of U2OS cells incubated with indicated fatty acid (50 µM) for 1 h, washed and incubated with of BODIPY-tetrazine (10 µM) for indicated time, then washed and imaged.

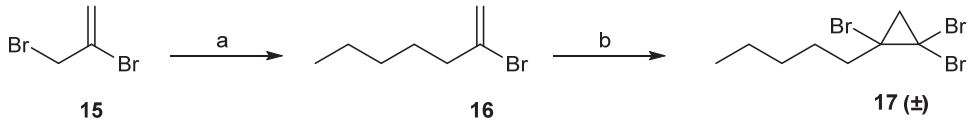
Synthesis of cyclopropene-arachidonic acid **2**

In order to expand this approach to study the derivatives of arachidonic acid, cyclopropene-arachidonic acid was synthesized. To obtain the desired cyclopropene-arachidonic acid (**2**), two fragments were assembled and joined. For the first fragment, the three unmodified double bonds were generated by partial hydrogenation of skipped alkynes. The second fragment contained the cyclopropene which was introduced in the final step. The synthesis commenced with esterification of hex-5-ynoic acid (**6**) using acidic MeOH which afforded compound **7** in quantitative yield (Scheme 1). Then, but-2-yne-1,4-diol (**8**) was tosylated and purified by crystallization of disubstituted byproduct to obtain compound **9** in a yield of 56%. A copper-mediated cross-coupling between compounds **7** and **9** led to skipped alkyne **10** in a yield of 77%. Compound **10** was activated for further elongation via tosylation leading to unstable **11**. Subsequent cross-coupling of this sulfonate ester with prop-2-yn-1-ol afforded skipped alkyne **12** in a moderate yield of 32% over two steps. This was immediately partially hydrogenated using P-2 nickel boride catalyst[32,33] to obtain the more stable triene **13** in 46% yield, which was then saponified to obtain carboxylic acid **14** in 87% yield.



Scheme 1 | Synthesis of (5*Z*,8*Z*,11*Z*)-13-hydroxytrideca-5,8,11-trienoic acid 14. Reagents and conditions: (a) $SOCl_2$, MeOH, 0 °C to rt, 16 h, quant.; (b) tosyl chloride, pyridine, DCM, 0 °C to 10 °C, 2 h, 62%; (c) CuI, NaI, K_2CO_3, DMF, rt, 16 h, 77%; (d) tosyl chloride, pyridine, $CHCl_3$, 0 °C to rt, 2 h; (e) prop-2-yn-1-ol, CuI, NaI, K_2CO_3, DMF, rt, 16 h, 32% over two steps; (f) H_2, $Ni(OAc)_2{\cdot}4H_2O$, $NaBH_4$, ethylenediamine, EtOH, rt, 4 h, 46%; (g) 1 M aq. LiOH, THF, rt, 16 h, 87%.

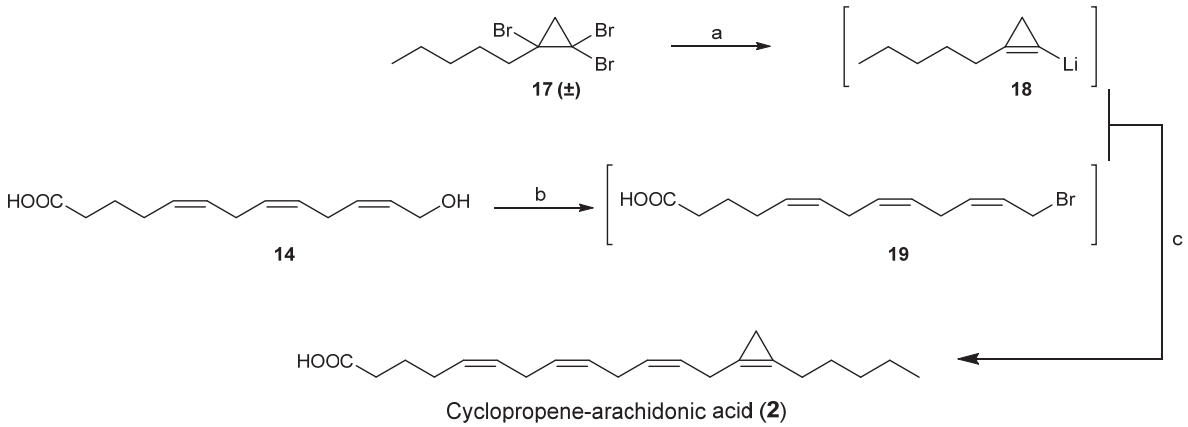
To obtain the cyclopropene fragment, 2,3-dibromopro-1-ene (**15**) was substituted by butylmagnesium bromide to obtain 2-bromohept-1-ene **16** in 69% yield after purification by distillation (Scheme 2). Cyclopropanation of **16** with dibromocarbene formed by deprotonation of bromoform was achieved under phase transfer conditions to afford the cyclopropane **17** in 36% yield.



Scheme 2 | Synthesis of 1,1,2-tribromo-2-pentylcyclopropane 17. Reagents and conditions: (a) Mg, 1,2-dibromoethane, 1-bromobutane, Et_2O, reflux, 15 min; then 2,3-dibromoprop-1-ene, CuCl, Et_2O, reflux, 4 h, 79%; (b) $CHBr_3$, cetyltrimethylammonium bromide, aq. NaOH (50% w/v), rt, 16 h, 36%.

To complete the synthesis of the desired cyclopropene fatty acid **2**, two unstable intermediates were generated and conjoined (Scheme 3). Lithiocyclopropene **18** was formed by a 1,2-dihaloelimination followed by a metal-halogen exchange reaction using 2 equivalents of *n*-BuLi at -78 °C in dry Et_2O, leading to the intermediate nucleophile.[34] The electrophilic allyl bromide **19** was generated using *N*-bromosuccinimide (NBS) and dimethyl sulfide under mild conditions, but cyclized over time and had to be used immediately.

However, attempts to substitute allyl bromide **19** resulted mainly in intramolecular cyclization, due to high dilution of the reactants and poor nucleophilicity of lithiocyclopropene **18**.[35] As hexamethylphosphoramide (HMPA) is known to affect organolithium aggregates and increase their nucleophilicity when used as an additive,[36] the freshly formed solution of lithiocyclopropene **18** was concentrated to a reduced volume under a flow of nitrogen gas, and the electrophilic allyl bromide **19** was added as solution in HMPA. This resulted in conversion to the desired cyclopropene-arachidonic acid **2** which was obtained in a yield of 28% after purification as judged by mass. The purity was between 80-90% as estimated by ^{1}H NMR, but attempts at further purifications were unsuccessful and resulted in major loss of the product.



Scheme 3 | Synthesis of cyclopropene-arachidonic acid (2). Reagents and conditions: (a) *n*-BuLi, Et_2O, -80 °C to rt, 25 min; (b) NBS, dimethyl sulfide, DCM, -25 °C to rt, 1 h; (c) HMPA, Et_2O, 0 °C to rt, 15 min, 28%.

Conclusion

In this chapter, the first use of 1,2-substituted cyclopropenes for live-cell imaging is reported. Plant-derived sterculic acid was compared to oleic acid and showed excellent fluorescent signal after reaction with tetrazine-quenched BODIPY **5**, whereas cells treated with oleic acid showed minimal background fluorescence. This demonstrates the possibility of substituting a fatty acid alkene for a cyclopropene to track lipid localization in live cells. Furthermore, the synthesis of a cyclopropene-tagged arachidonic acid is described which can be used to study arachidonic acid and its derivatives. However, the resulting cyclopropene proved to be unstable. Cyclopropenes are known to undergo ene reactions with alkenes, which could explain the tendency for degradation under solvent- and oxygen-free conditions observed for cyclopropene **2**.[37,38] It is recommended that further research on cyclopropene **2** minimizes the number of synthetic steps to obtain its derivatives. Nevertheless, as degradation products of the cyclopropene do not participate in the IEDDA reaction, cyclopropene-modified versions of arachidonic acid derivatives are in principle suitable for investigation of arachidonic acid and metabolites by microscopy.

Experimental procedures

General

Oleic acid (**3**, Cayman Chemicals) and sterculic acid (**4**, Matreya, SKU 1236) were stored as 10 mM DMSO solutions under nitrogen at -80 °C. BODIPY-tetrazine **5** was a kind gift from Alexi Sarris (Leiden University) and stored as 10 mM DMSO solution at -20 °C. All cell culture disposables were from Sarstedt.

Cell culture and imaging

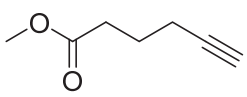
U2OS cells were cultured at 37 °C under 7% CO_2 in DMEM (Sigma Aldrich, D1145) containing stable glutamine, 10% (v/v) New Born Calf Serum (Thermo Fisher) and penicillin and streptomycin (200 µg/mL each, Duchefa). Cells were passaged twice a week at 80-90% confluence by trypsinization and resuspension in fresh medium. Cell lines were purchased from ATCC and were tested regularly for mycoplasma contamination. Cultures were discarded after 2-3 months of use.

$2.0x10^4$ U2OS cells were plated in 12-well plates 24 h prior to microscopy experiments. Cells were treated with 600 µL of 50 µM oleic acid (**3**) or sterculic acid (**4**) in phenol red free DMEM with serum for 1 h at 37 °C under 7% CO_2. The medium was aspirated, cells were washed twice with PBS and treated with tetrazine-BODIPY (**5**) in phenol red free DMEM with serum for the indicated incubation time at 37 °C under 7% CO_2. Medium was aspirated, cells were washed three times with PBS and 600 µL of PBS was added for imaging, which was done using a EVOS M7000 Imaging System (Thermo Fisher).

Synthesis

Dry solvents were prepared by storage on activated 4 Å molecular sieves for at least 24 h. The reactions were performed under an inert atmosphere of nitrogen gas unless stated otherwise. All reagents were purchased from Alfa Aesar, Sigma Aldrich/Merck or Acros and used without further purification. Flash column chromatography was performed using SiliCycle silica gel type SiliaFlash P60 (230-400 mesh). TLC analysis was performed on Merck silica gel 60/Kieselguhr F254, 0.25 mm. Compounds were visualized using $KMnO_4$ stain (K_2CO_3 (40g), $KMnO_4$ (6 g), and water (600 mL)). ^1H NMR and ^{13}C NMR spectra were recorded on a Bruker AV-400 (400 MHz) or AV-500 (500 MHz) spectrometer. Chemical shift values are reported in ppm relative to the tetramethylsilane signal for ^1H NMR (δ = 0 ppm) and relative to the solvent signal of $CDCl_3$ for ^{13}C NMR (δ = 77.16 ppm). Data are reported as follows: Chemical shifts (δ), multiplicity (s = singlet, d = doublet, dd = doublet of doublets, ddt = doublet of doublet of triplets, td = triplet of doublets, t = triplet, q = quartet, p = pentet, bs = broad singlet, m = multiplet), coupling constants *J* (Hz), and integration. High-resolution mass spectra (HRMS) were recorded on a Thermo Scientific Q Exactive HF Orbitrap Mass Spectrometer equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas low 10, capillary temperature 275 °C) with resolution R = 60,000 at m/z = 400 (mass range = 150-1,500).

Methyl hex-5-ynoate (7)



To a cooled (0 °C) solution of hex-5-ynoic acid (5.19 g, 46.3 mmol) in dry MeOH (160 mL) was added thionyl chloride (3.70 mL, 50.9 mmol) dropwise over 10 min under vigorous stirring and the reaction was allowed to reach rt overnight. The reaction was diluted with DCM (250 mL) and quenched with sat. aq. $NaHCO_3$ (300 mL). The layers were separated and the aq. layer was extracted with DCM (2 x 250 mL). The combined organic layers were dried over $MgSO_4$, filtered and concentrated under reduced pressure to afford the title compound as a pale yellow oil (5.82 g, 46.3 mmol, quant.). R_f = 0.7 (Et_2O/pentane = 1:3). ^1H NMR (400 MHz, $CDCl_3$) δ 3.68 (s, 3H), 2.46 (t, *J* = 7.4 Hz, 2H), 2.26 (td, *J* = 6.9, 2.6 Hz, 2H), 1.97 (t, *J* = 2.6 Hz, 1H), 1.91 – 1.79 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.67, 83.38, 69.25, 51.74, 32.78, 23.72, 17.98. HRMS: Calculated for $[C_7H_{10}O_2+H]^+$ 127.0754, found 127.0755.

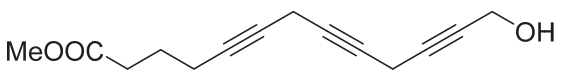
4-Hydroxybut-2-yn-1-yl 4-methylbenzenesulfonate (9)

To a cooled (0 °C) solution of 2-butyne-1,4-diol (10.0 g, 116 mmol) in dry DCM (210 mL) was added pyridine (18.9 mL, 232 mmol) and tosyl chloride (20.0 g, 105 mmol) in small portions over a period of 10 min. The reaction was stirred for 1 h and then at 10 °C for 1 h. The mixture was diluted with H_2O (100 mL) and 3 M aq. HCl was added until acidic (pH <2). The layers were separated and the organic layer was washed with 1 M aq. HCl (120 mL) and brine (60 mL). The organic layer was dried over $MgSO_4$, filtered and concentrated under reduced pressure. The mixture was dissolved in hot MeOH (50 mL) and cooled to -30 °C overnight. The mixture was filtered and rinsed with ice-cold MeOH (30 mL). The filtrate was concentrated under reduced pressure to afford the title compound as a yellow oil (15.7 g, 65.3 mmol 62%). R_f = 0.6 (pentane/EtOAc = 1:1). ^1H NMR (400 MHz, $CDCl_3$) δ 7.81 (d, *J* = 8.3 Hz, 2H), 7.39 – 7.32 (d, *J* = 7.8 Hz, 2H), 4.73 (t, *J* = 1.8 Hz, 2H), 4.16 (t, *J* = 1.9 Hz, 2H), 2.45 (s, 3H), 1.92 (bs, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 129.98, 129.97, 128.30, 128.29, 57.99, 50.86, 21.81. HRMS: Calculated for $[C_{11}H_{12}O_4S+NH_4]^+$ 258.0795, found 258.0791.

Methyl 10-hydroxydeca-5,8-diynoate (10)

To a mixture of CuI (1.095 g, 10.00 mmol), NaI (1.499 g, 10.00 mmol) and K_2CO_3 (1.383 g, 10.00 mmol) was added nitrogen-purged DMF (20 mL) and the mixture was stirred for 15 min. Then, methyl hex-5-ynoate **7** (1.262 g, 10 mmol) dissolved in nitrogen-purged DMF (5 mL) was added to the reaction mixture. Sulfonate ester **9** (2.64 g, 11.0 mmol) dissolved in nitrogen-purged DMF (5 mL) was added dropwise to the reaction over 10 min and the reaction was stirred overnight in the dark. The reaction was diluted in Et_2O (50 mL), cooled to 0 °C and quenched with sat. aq. NH_4Cl (2.0 mL). It was stirred at room temperature for 30 min, followed by gravity filtration over a celite/sand pad. The pad was rinsed with Et_2O (1 L) and the filtrate was concentrated under reduced pressure to a reduced volume (100 mL). The organic layer was washed with sat. aq. NH_4Cl (10 mL) and H_2O (50 mL). The aqueous layer was extracted with Et_2O (3 x 100 mL) and the combined organic layers were washed with brine (5 x 200 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure. Purification of the residue by column chromatography (Et_2O/pentane = 1:9 to Et_2O) afforded the title compound as a yellow oil (1.50 g, 7.74 mmol, 77%). R_f = 0.3 (Et_2O/pentane = 1:1). ^1H NMR (400 MHz, $CDCl_3$) δ 4.25 (t, *J* = 2.1 Hz, 2H), 3.67 (s, 3H), 3.22 - 3.13 (m, 2H), 2.43 (t, *J* = 7.5, 2H), 2.23 (tt, *J* = 6.9, 2.3 Hz, 2H), 1.90 – 1.74 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.84, 80.64, 79.89, 78.66, 74.57, 51.77, 51.34, 32.99, 23.90, 18.27, 9.95. HRMS: Calculated for $[C_{11}H_{14}O_3+H]^+$ 195.1016, found 195.1019.

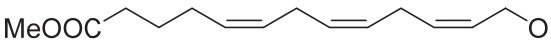
Methyl 13-hydroxytrideca-5,8,11-triynoate (12)



To a cooled (0 °C) solution of alcohol **10** (4.822 g, 24.83 mmol) in $CHCl_3$ (50 mL) was added pyridine (6.02 mL, 74.5 mmol) and subsequently tosyl chloride (9.47 g, 49.7 mmol) in portions over 15 min. The reaction was stirred at rt for 2 h. The reaction mixture was then cooled to 0 °C and diluted with Et_2O (150 mL) and H_2O (80 mL). The layers were separated

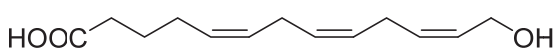
and the organic layer was washed with 1 M aq. HCl (80 mL), sat. aq. $NaHCO_3$ (80 mL) and brine (2 x 80 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure. Column chromatography of the residue (Et_2O/pentane = 1:9 to Et_2O) afforded sulfonate ester **11**, which was used immediately due to its instability. To a mixture of CuI (4.73 g, 24.8 mmol), NaI (3.72 g, 24.8 mmol) and K_2CO_3 (3.43 g, 24.8 mmol) was added nitrogen-purged DMF (25 mL) and the mixture was stirred for 15 min. Prop-2-yn-1-ol (1.67 g, 29.8 mmol) dissolved in nitrogen-purged DMF (20 mL) was added and the mixture was stirred for 30 min. A solution of sulfonate ester **11** in nitrogen-purged DMF (30 mL) was added to the reaction dropwise over 10 min and the reaction was stirred overnight in the dark. It was then diluted with Et_2O (80 mL), quenched with sat. aq. NH_4Cl (6 mL) and stirred for 30 min at rt. The mixture was filtered over celite and the filter was rinsed with Et_2O (1 L). The filtrate was concentrated under reduced pressure to a reduced volume (100 mL). The organic layer was washed with sat. aq. NH_4Cl (30 mL) and H_2O (100 mL). The combined aqueous layers were extracted with Et_2O (3 x 150 mL) and the combined organic layers were washed with brine (5 x 100 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure. Purification of the residue by column chromatography (Et_2O/pentane = 1:4 to 2:1) afforded the title compound as an orange oil (1.85 g, 7.97 mmol, 32%). R_f = 0.5 (Et_2O/pentane = 1:1). ^1H NMR (400 MHz, $CDCl_3$) δ 4.26 (t, *J* = 2.1 Hz, 2H), 3.68 (s, 3H), 3.20 (p, *J* = 2.3 Hz, 2H), 3.12 (p, *J* = 2.4 Hz, 2H), 2.44 (t, *J* = 7.5 Hz, 2H), 2.23 (tt, *J* = 6.9, 2.4 Hz, 2H), 1.87 – 1.76 (p, *J* = 7.6 Hz, 2H), 1.68 (bs, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.87, 79.73, 78.88, 75.78, 75.45, 74.81, 73.97, 51.76, 51.38, 33.01, 23.95, 18.31, 10.03, 9.88. HRMS: Calculated for $[C_{14}H_{16}O_3+H]^+$ 233.1172, found 233.1176.

Methyl (5*Z*,8*Z*,11*Z*)-13-hydroxytrideca-5,8,11-trienoate (13)



In a Schlenk flask, nickel acetate hydrate (0.214 g, 0.861 mmol) and 96% EtOH (7 mL) were placed under H_2 atmosphere. Sodium borohydride (0.033 g, 0.861 mmol) in 96% EtOH (2 mL) was added over 40 s and stirred at room temperature for 20 min. Freshly distilled ethylenediamine (0.174 mL, 2.58 mmol) was added and the reaction was stirred for 10 min. Compound **12** (0.50 g, 2.15 mmol) in 96% EtOH (3 mL) was added and the reaction was stirred under H_2 atmosphere for 5 h. The reaction was then quenched with NH_4Cl (2 g) and was stirred for 15 min under N_2 atmosphere. The mixture was filtered over celite and the pad was rinsed with EtOH (500 mL). The filtrate was concentrated under reduced pressure. The residue was dissolved in Et_2O (100 mL) and washed with sat. aq. NH_4Cl (100 mL). The aq. layer was extracted with Et_2O (2 x 100 mL) and the combined organic layers were washed with brine (2 x 100 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure. Purification of the residue by column chromatography (Et_2O/pentane = 1:4 to 1:2) afforded the title compound as a colorless oil (0.234 g, 0.98 mmol, 46%). R_f = 0.7 (Et_2O/pentane = 3:1). ^1H NMR (500 MHz, $CDCl_3$) δ 5.67 – 5.49 (m, 2H), 5.43 – 5.33 (m, 4H), 4.25 – 4.21 (m, 2H), 3.67 (s, 3H), 2.86 (t, *J* = 6.6 Hz, 2H), 2.79 (t, *J* = 6.0 Hz, 2H), 2.33 (td, *J* = 7.5, 1.2 Hz, 2H), 2.14 – 2.07 (m, 2H), 1.70 (m, 2H), 1.47 (bs, 1H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 130.98, 129.19, 128.97, 128.83, 128.80, 127.67, 58.70, 51.68, 33.57, 26.71, 25.97, 25.75, 24.88. HRMS: Calculated for $[C_{14}H_{22}O_3+Na]^+$ 261.1461, found 261.1470.

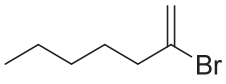
(5*Z*,8*Z*,11*Z*)-13-Hydroxytrideca-5,8,11-trienoic acid (14)



To a solution of triene **13** (0.423 g, 1.776 mmol) in THF (16 mL) was added 1 M aq. LiOH (16 mL) and the reaction was stirred overnight. The reaction mixture was cooled to 0 °C and diluted with Et_2O (50 mL). 1 M aq. HCl was added until acidic (pH <2) and NaCl was added to saturation. The aq. layer was extracted with Et_2O (3 x 50 mL). The combined organic layers were dried over Na_2SO_4, filtered and concentrated under reduced pressure. Purification of the residue by column chromatography (MeOH/DCM = DCM to 1:49) afforded the title compound **7** (0.346 g, 1.54 mmol, 87%) as a colorless oil. R_f = 0.2 (MeOH/DCM = 5:95). ^1H NMR (400 MHz, $CDCl_3$) δ 6.67 (bs, 2H), 5.70 – 5.50 (m, 2H), 5.48 – 5.28 (m, 4H), 4.24 (d, *J* = 6.8 Hz, 2H), 2.87 (d, *J* = 6.9 Hz, 2H), 2.81 (t, *J* = 6.1 Hz, 2H), 2.36 (t, *J* = 7.0 Hz, 2H), 2.14 (q, *J* = 7.5 Hz, 2H), 1.70 (p, *J* = 7.0 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 131.65,

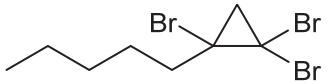
129.01, 129.00, 128.91, 128.26, 127.48, 58.49, 33.16, 26.52, 25.95, 25.80, 24.61. HRMS: Calculated for $[C_{13}H_{20}O_3+Na]^+$ 247.1305, found 247.1310.

2-Bromohept-1-ene (16)



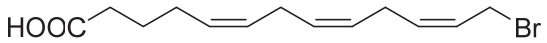
Magnesium (24.31 g, 1.00 mol) was placed in dry Et_2O (133 mL) and 1,2-dibromoethane (17 µL, 0.20 mmol) was added. The mixture was heated to reflux and 1-bromobutane (27.4 g, 21.5 mL, 200 mmol) was added while maintaining a gentle reflux over 15 min to form butylmagnesium bromide, which was titrated.[39] In a different flask, CuCl (0.621 g, 6.28 mmol) was suspended in dry Et_2O (120 mL) and 2,3-dibromoprop-1-ene (25.1 g, 126 mmol) was added. Butylmagnesium bromide (83 mL, 109 mmol) was then added dropwise using a cannula to maintain a gentle reflux. The reaction was stirred for 4 h, cooled to 0 °C and quenched using sat. aq. NH_4Cl (100 mL). The aq. layer was extracted using Et_2O (2 x 200 mL) and the combined organic layers were dried over Na_2SO_4, filtered and concentrated under reduced pressure. Vacuum distillation (72-79 °C, 86 mmHg) afforded the title compound as an orange liquid (15.25 g, 86 mmol, 79%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.55 (d, *J* = 1.4 Hz, 1H), 5.38 (d, *J* = 1.6 Hz, 1H), 2.41 (d, *J* = 7.6 Hz, 2H), 1.55 (p, *J* = 7.4 Hz, 2H), 1.37 – 1.23 (m, 4H), 0.90 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 135.10, 116.36, 41.52, 30.72, 27.71, 22.51, 14.13. The spectra were in full accordance with literature experimental data.[40]

1,1,2-Tribromo-2-pentylcyclopropane (17)



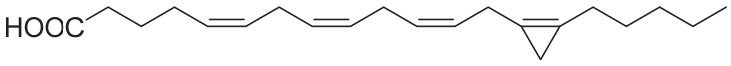
To a vigorously stirred mixture of compound **16** (3.238 g, 18.28 mmol), cetrimonium bromide (600 mg, 1.65 mmol) and bromoform (3.20 mL, 36.6 mmol) was added dropwise aq. NaOH (7.31 g, 50% (w/v)) over 10 min and the reaction was stirred overnight. The reaction was diluted with H_2O (100 mL) and extracted with DCM (3 x 150 mL). The combined organic layers were washed with brine (3 x 150 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure. Purification of the residue by column chromatography (pentane) and high vacuum afforded the title compound as a brown oil (2.30 g, 6.59 mmol, 36%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.11 – 1.95 (m, 2H), 1.95 (d, *J* = 9.2 Hz, 1H), 1.83 (d, *J* = 9.2 Hz, 1H), 1.80 – 1.61 (m, 2H), 1.42 – 1.27 (m, 4H), 0.92 (t, *J* = 6.9 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 46.01, 41.85, 38.22, 33.34, 31.27, 27.53, 22.68, 14.15. The spectra were in full accordance with literature experimental data.[40]

(5*Z*,8*Z*,11*Z*)-13-Bromotrideca-5,8,11-trienoic acid (19)



To a cooled (0 °C) mixture of NBS (0.089 g, 0.50 mmol) and dry DCM (2 mL) was added dimethyl sulfide (45 µL, 0.60 mmol) over 5 min. The reaction was stirred for 15 min and then cooled to -25 °C. A solution of compound **14** (0.075 g, 0.33 mmol) in dry DCM (2 mL) was added dropwise to the mixture over 10 min. The reaction was allowed to reach rt and was stirred for an hour. It was then diluted with DCM (20 mL) and H_2O (10 mL). The aq. layer was separated and extracted with DCM (2 x 20 mL). The combined organic layers were washed with brine (2 x 20 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure at rt to afford title compound as a colorless oil, which was used immediately in the next reaction due to its instability. Purification of an aliquot by column chromatography (MeOH/DCM = DCM to 1:99) allowed characterization by NMR. R_f = 0.6 (EtOAc/pentane = 1:1). ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.81 – 5.70 (m, 1H), 5.64 – 5.52 (m, 1H), 5.48 – 5.30 (m, 4H), 4.02 (d, *J* = 8.4 Hz, 2H), 2.95 – 2.89 (m, 2H), 2.86 – 2.79 (m, 2H), 2.41 – 2.34 (m, 2H), 2.19 – 2.10 (m, 2H), 1.72 (p, *J* = 7.6 Hz, 2H).

(5*Z*,8*Z*,11*Z*)-13-(2-pentylcycloprop-1-en-1-yl)trideca-5,8,11-trienoic acid (cyclopropene-arachidonic acid, 2)



To a cooled (-80 °C) solution of 1,1,2-tribromo-2-pentylcyclopropane **17** (233 mg, 0.668 mmol) in dry Et_2O (1 mL) was added *n*-BuLi (1.6 M in hexanes, 836 µL, 1.34 mmol). The reaction was allowed to reach rt and stirred for 25 min, after which a stream of N_2 gas was passed over to reduce the volume of solvent by half. The mixture was cooled to 0 °C and a solution of bromide **19** (53.3 mg, 0.186 mmol) in dry HMPA (1 mL) was added, after which the reaction was stirred for 15 min. The reaction was quenched with aq. citric acid (10% (w/v), 2 mL) and diluted with Et_2O (10 mL). The layers were separated and the aq. layer was extracted with Et_2O (10 mL). The combined organic layers were washed with sat. aq. LiCl (10 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure. Purification of the residue by column chromatography (Et_2O/pentane = 1:39 + 0.1% AcOH to 1:11 + 0.1% AcOH) afforded the title compound with minor impurities as a clear oil (16.2 mg, 0.051 mmol, 28%). Attempts at further purification resulted in near complete degradation. R_f = 0.5 (EtOAc/pentane = 1:3). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.60 – 5.51 (m, 1H), 5.51 – 5.32 (m, 5H), 3.17 (d, *J* = 6.091 Hz, 2H), 2.85 (t, *J* = 6.553 Hz, 2H), 2.80 (t, *J* = 5.850 Hz, 2H), 2.42 – 2.33 (m, 5H), 2.18 – 2.09 (m, 2H), 1.77 – 1.67 (m, 2H), 1.59 – 1.50 (m, 2H), 1.34 – 1.29 (m, 3H), 0.89 (t, *J* = 6.726 Hz, 5H), 0.83 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 180.14, 129.16, 128.88, 128.35, 128.18, 125.82, 111.69, 107.83, 33.53, 31.74, 27.24, 26.58, 26.04, 25.80, 25.74, 24.79, 24.61, 22.61, 14.21, 7.84. LC-MS: Calculated for $[C_{21}H_{32}O_2+H]^+$ 317.3, found 317.1.

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