Photo-Release of 2-Arachidonoylglycerol in Live Cells.

Aurélien Laguerre[1], Sebastian Hauke[2], Jian Qiu[1], Martin J. Kelly[1] and Carsten Schultz*[1, 2]

[1]Oregon Health and Science University, Dept. Chemical Physiology & Biochemistry, Portland, OR, USA.

[2] European Molecular Biology Laboratory, Cell Biology and Biophysics Unit, 69117 Heidelberg, Germany.

-- Supporting Information –

Part 1. Synthetic chemistry

General. All chemicals were obtained from commercial sources (Acros, Sigma, Aldrich, Enzo, Calbiochem, Lancaster or Merck) and were used without further purification unless otherwise specified. Diazoxide (7-chloro-3-methyl-2H-1,2,4-benzo-thiadiazin 1,1- dioxide) from Tocris was dissolved in dimethylsulfoxide (DMSO) to a stock concentration of 300 mM and diluted 1,000 fold to a working concentration of 300 μM. Solvents for chromatography (HPLC grade) were obtained from VWR and dry solvents were obtained from Sigma. Deuterated solvents were obtained from Deutero GmbH, Karlsruhe, Germany. All reactions were carried out using dry solvents under an inert atmosphere unless otherwise stated in the respective experimental procedure. TLC was performed on precoated plates of silica gel (Merck, 60 F254) using UV light (254 or 366 nm) or a solution of $KMNO_4$ in H_2O (1.5 g $KMnO_4$, 10 g K_2CO_3 and 1.25 mL 10% NaOH in 200 mL of H_2O) for analysis. HPLC was performed on a 1260 Infinity system from Agilent Technologies equipped with an EC 250/4 NUCLEODUR 100-5 C18ec analytical column. HPLC-MS spectra were recorded on a Q-Exactive Plus (ThermoFisher) high-resolution mass spectrometer (HRMS)

coupled with a Vanquish UHPLC system (ThermoFisher). ^{1}H- and ^{13}C-NMR spectra were measured on a 400 MHz Bruker UltraShield^{TM} spectrometer. Chemical shifts of ^{1}H- and ^{13}C-NMR-spectra are referenced indirectly to tetramethylsilane, chemical shifts are given in ppm. Splitting patterns are designated as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; b, broad. ^{13}C-NMR-spectra were broadband hydrogen decoupled. Mass spectra (ESI) were recorded using an Advion Expressions CMS mass spectrometer.

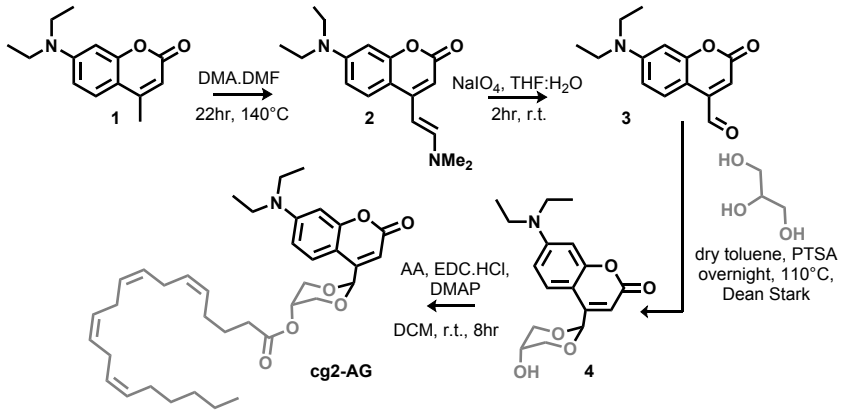


Figure S1: Four step chemical pathway for the synthesis of cg2-AG.

Chemical synthesis. The dimethylamino vinyl intermediates **2** and 7-diethylamino-4-formyllcoumarin **3** were synthesized by reproducing the methodology recently reported by Walter *et al*. [1]. Briefly, 7-diethylamino-4-methyl-2-oxo-2H-chromen (11.56 g, 50 mmol) and N,N-dimethylformamide dimethylacetal (10 mL, 75 mmol) in anhydrous N,N-dimethylformamide (20 mL) were stirred at 140°C for 22 h under an argon atmosphere. After allowing to cool to room temperature, volatiles were removed under reduced pressure. The residue was triturated with cyclohexane (20 mL) and filtrated. The filter cake was suspended in acetone (50 mL), filtrated and washed with two portions of acetone (2 × 25 mL). Compound **2** was obtained as a yellow solid (10 g, 34.9 mmol, 69.8%). Sodium (meta)periodate (22.4 g, 105 mmol) was added to a stirring suspension of **2** (10 g, 35 mmol) in tetrahydrofuran:water (1:1, 500 mL). After 2 h, solids were removed by filtration and washed with ethyl acetate (300 mL). The organic layer was separated, washed twice with a saturated sodium bicarbonate solution (2 × 200 mL), dried over Na_2SO_4) filtrated and evaporated under reduced pressure. The black residue obtained was dissolved in dichloromethane and dried under high vacuum to afford compound **3** (8.3 g, 33.8 mmol, 96.9%). All characterizations fully agreed with the reported procedure, see [1].

In a round bottom flask, intermediate **3** (1.5 g, 6.1 mmol), glycerol (567 µL, 5.1 mmol) and p-toluene sulfonic acid (2 mg, cat. amount) were added in dry toluene (35 mL). The mixture was stirred overnight under reflux conditions using a Dean Stark apparatus. After completion of the reaction, the crude mixture was poured into a separation funnel containing ethyl acetate (75mL) and 75 mL of distilled water. After separation of the two phases, the organic phase was washed with saturated $NaHCO_3$ solution, water and brine. The crude was purified by a first column chromatography on silica using cyclohexane-acetone (2:1) as eluent. The two isolated acetal isomers were separated by a second column chromatography on silica using cyclohexane-AcOEt (1:2) as eluent, providing the desired alcohol **4** (320 mg, 1 mmol, 16 %). ^{1}H NMR 400MHz, $CDCl_3$, δ (ppm): 7.50 (d, 1H), 6.55 (d, 1H), 6.47 (s, 1H), 6.32 (s, 1H), 5.63 (s, 1H), 4.20 (dd, 4H), 3.69 (s, 1H), 3.39 (q, 4H), 3.06 (s, 1H), 1.17 (t, 1H). ^{13}C NMR, $CDCl_3$, δ (ppm): 162.27, 156.78, 150.40, 149.26, 126.09, 108.73, 106.87, 105.60, 97.96, 97.72, 72.30, 63.86, 44.79, 12.44. HRMS (ESI) m/z = 320.14847 $[M+H]^+$, 320.14928 calculated for $C_{17}H_{22}NO_5^+$

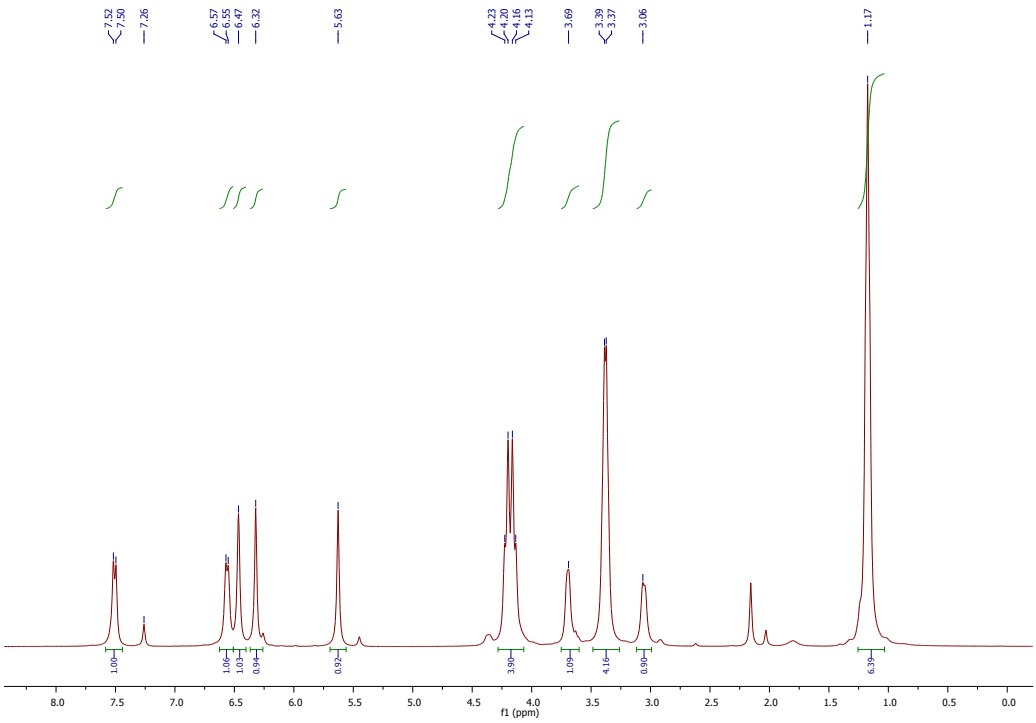


Figure S2: ^{1}H NMR spectrum of alcohol **4** in $CDCl_3$

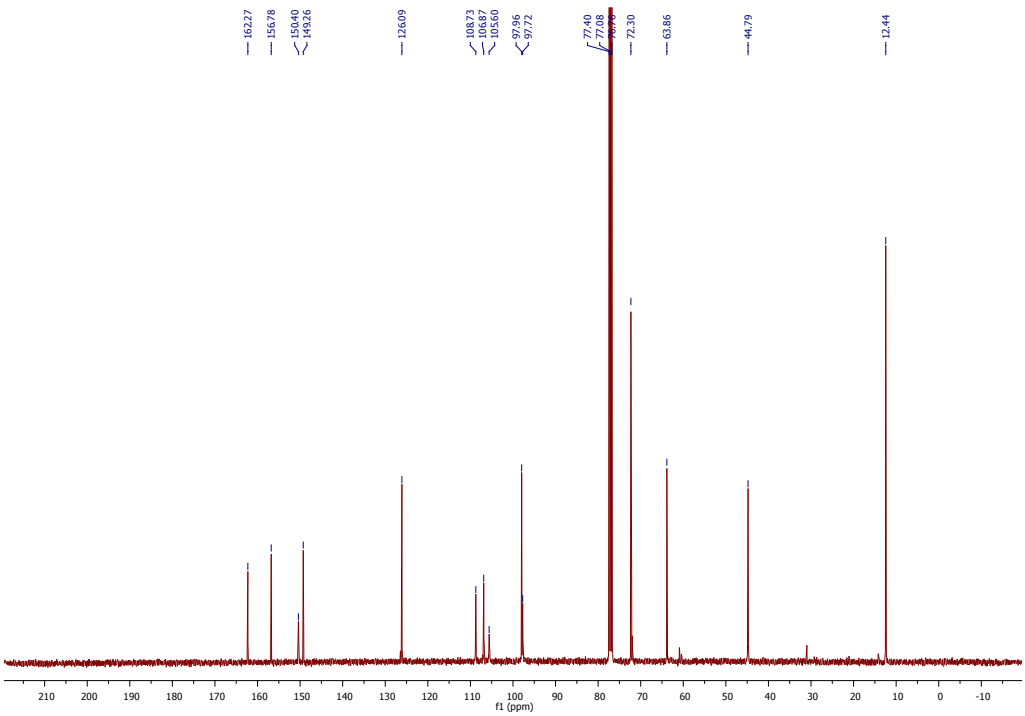


Figure S3: ^{13}C NMR spectrum of alcohol **4** in $CDCl_3$

Under an inert atmosphere, DMAP (1 mg, 7.9 μmol) and EDC.HCl (54 mg, 282 μmol) were added to a solution of arachidonic acid (57 mg, 62 μL, 188 μmol) in dry DCM (1.5 mL) and the solution was stirred at 0°C for 5 min. A solution of compound **4** (30 mg, 94 μmol) in dry CH_2Cl_2 (0.75 mL) was added dropwise into the arachidonic acid mixture. The solution was allowed to warm to room temperature and was stirred overnight. After removal of all volatiles under reduced pressure, the crude was purified by column chromatography on silica using CH_2Cl_2 /acetone (3:1) as eluent to afford **cg2-AG** (42 mg, 70 μmol, 74 %). ^1H NMR 400MHz, $CDCl_3$, δ (ppm): 7.72 (d, 1H), 6.65 (d, 1H), 6.49 (s, 1H), 6.26 (s, 1H), 5.59 (s, 1H), 5.37 (m, 8H), 4.77 (s, 1H), 4.16/4.31 (dd, 4H), 3.40 (q, 4H), 2.79 (m, 6H), 2.49 (t, 2H), 2.03 (q, 2H), 1.78 (m, 2H), 1.21 (m, 6H), 1.17 (t, 6H), 0.87 (t, 3H). ^{13}C NMR, $CDCl_3$, δ (ppm): 173.36, 162.12, 156.92, 149.09, 130.50, 129.09, 128.75, 128.58, 128.29, 128.07, 127.83, 127.53, 126.80, 98.98, 69.03, 65.03, 33.81, 33.35, 31.51, 29.32, 27.21, 26.54, 25.61, 24.75, 22.58, 14.09, 12.41. HRMS (ESI) m/z = 606.37744 $[M+H]^+$, 606.37891 calculated for $C_{37}H_{52}NO_6^+$.

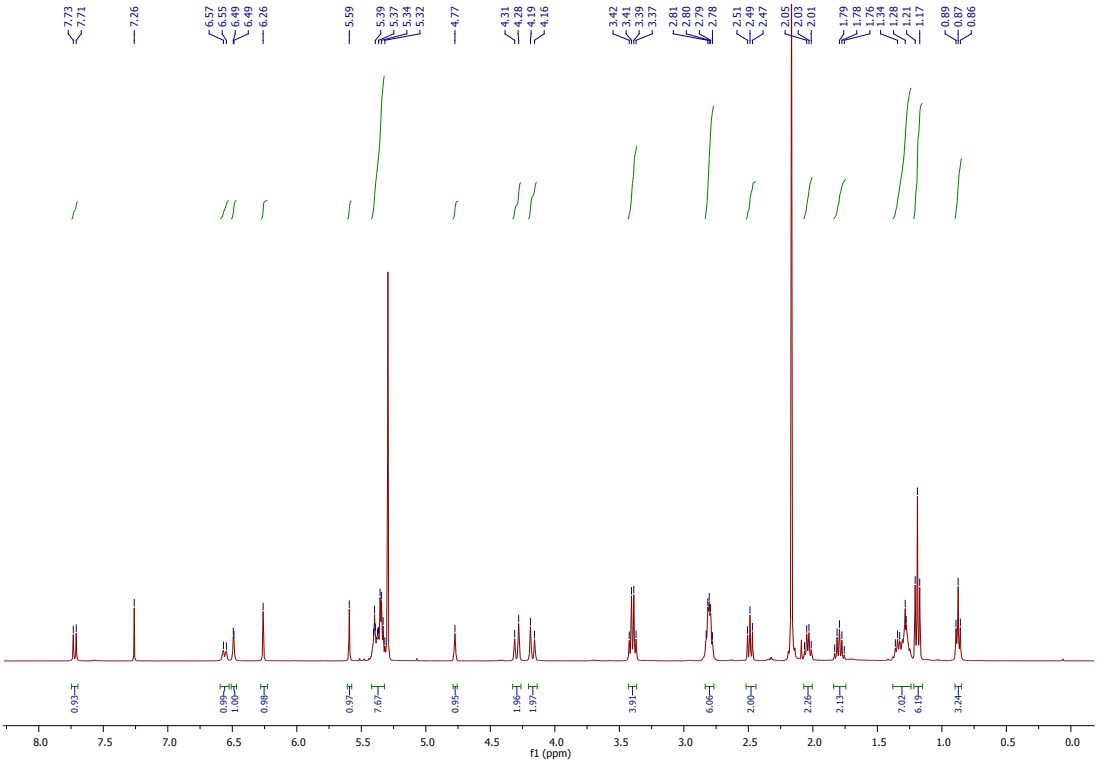


Figure S4: ^{1}H NMR spectrum of cg2-AG in $CDCl_3$

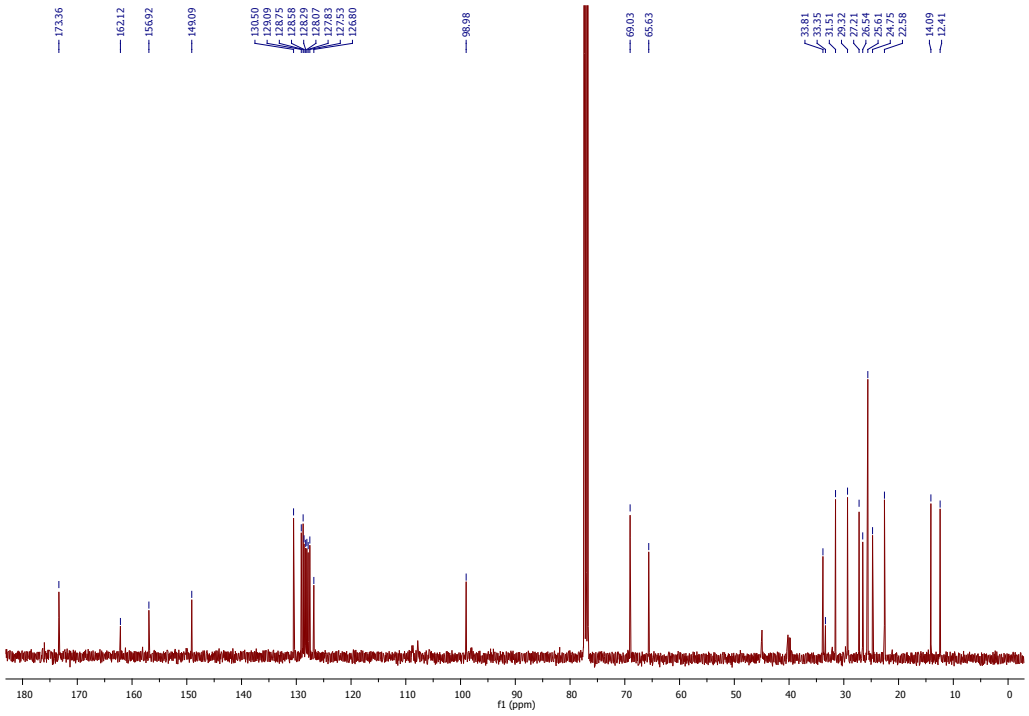


Figure S5: ^{13}C NMR spectrum of cg2-AG in $CDCl_3$

Part 2. Uncaging experiments

NMR analysis.

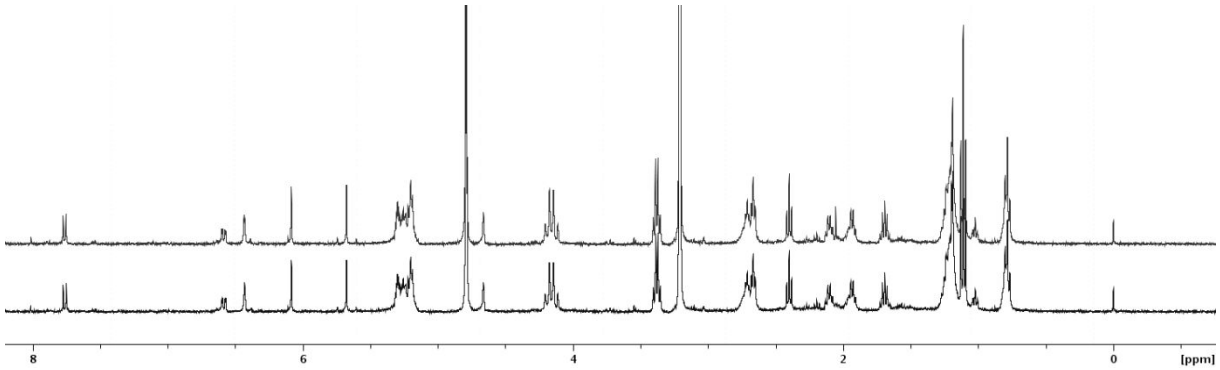


Figure S6: ^{1}H NMR spectra of cg2-AG in MeOD-*d4* before (upper trace) and after (lower trace) 15 min UV light illumination with a Newport 66924 light source equipped with a 1 kW xenon lamp and a 350 nm highpass filter.

HPLC-MS analysis.

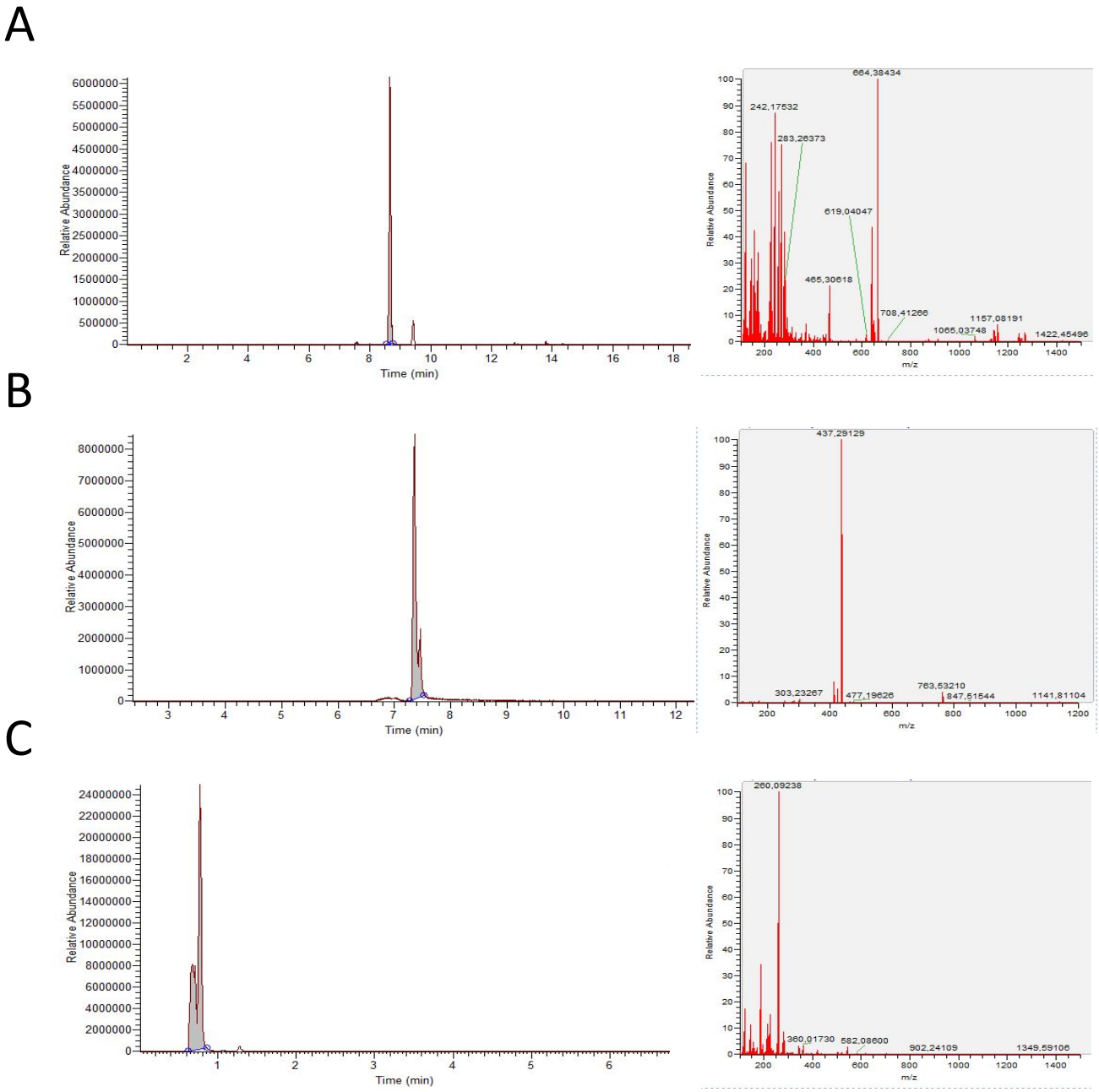


Figure S7: Analysis of cg2-AG, 2-AG or aldehyde **3** in aqueous solution by HPLC-MS. The mobile phase consisted of solvents A (acetonitrile−water (6:4)) and B (isopropyl alcohol−acetonitrile (9:1)), buffered with 10 mM ammonium acetate and 0.1% formic acid. Gradient: 20 to 100 % of B in 10 min, then back to 20% within 5 min. The MS parameters in the Tune software (Thermo Scientific) were set as: spray voltage 4 kV, sheath gas 30 and auxiliary gas 5 units, S-Lens 65 eV, capillary temperature 320 °C and vaporization temperature of auxiliary gas 300 °C. Xcalibur Quan software (Thermo Scientific) was used for data analysis, including the generation of

extracted ion chromatograms (XIC) and raw data visualization. **A.** HRMS (ESI, negative mode) cg2-AG, m/z = 664.38452 $[M+acetate]^-$, 664.38549 calculated for $C_{39}H_{54}NO_8^-$. **B.** 2-AG, m/z = 437.29129 $[M+acetate]^-$, 437.29086 calculated for $C_{25}H_{41}O_6^-$ and **C.** Aldehyde **3** (under these buffer conditions, the carboxylic acid form appeared to be the main peak) m/z = 260.09238 $[M-H]^-$, 260.09283 calculated for $C_{14}H_{14}NO_4^-$

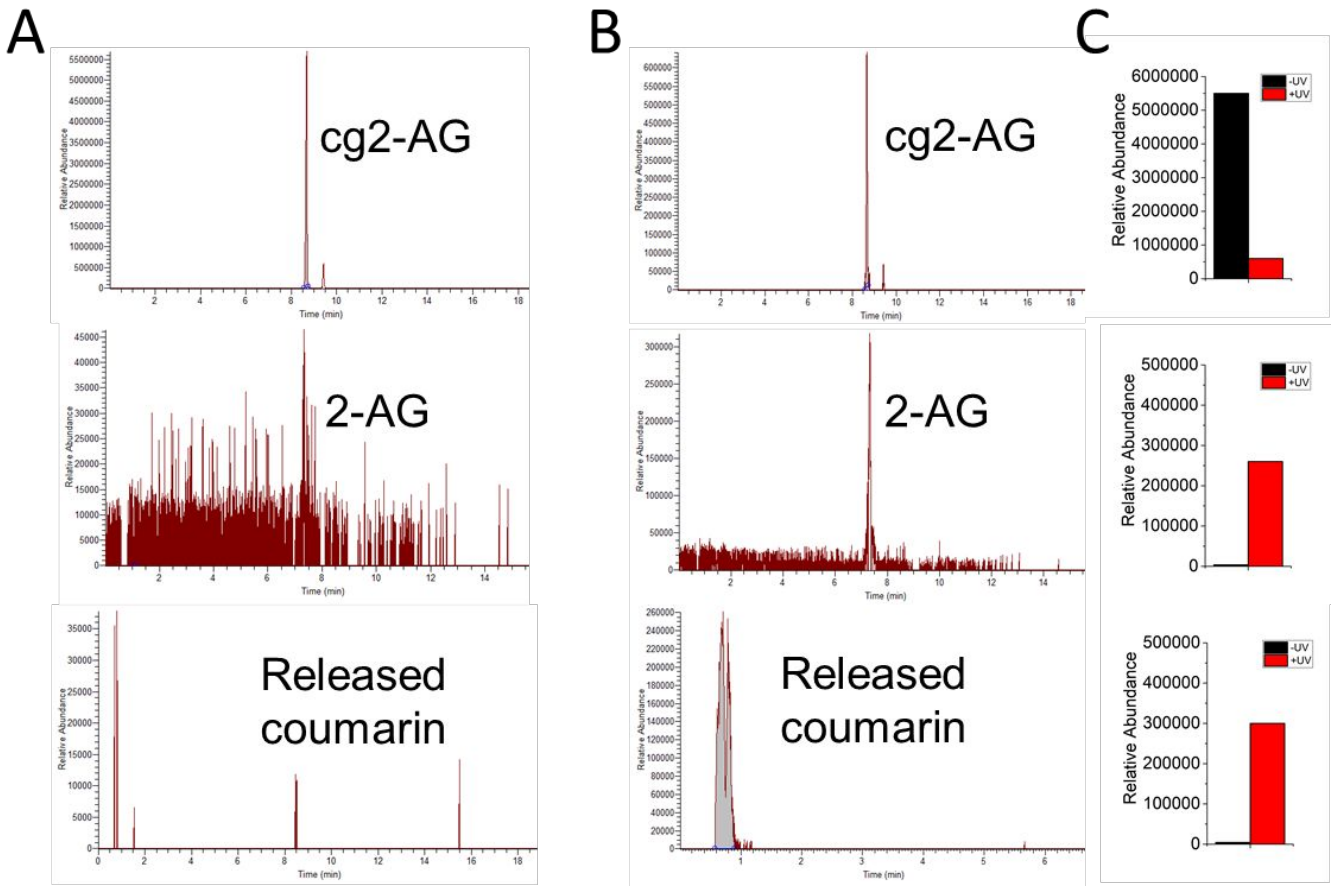
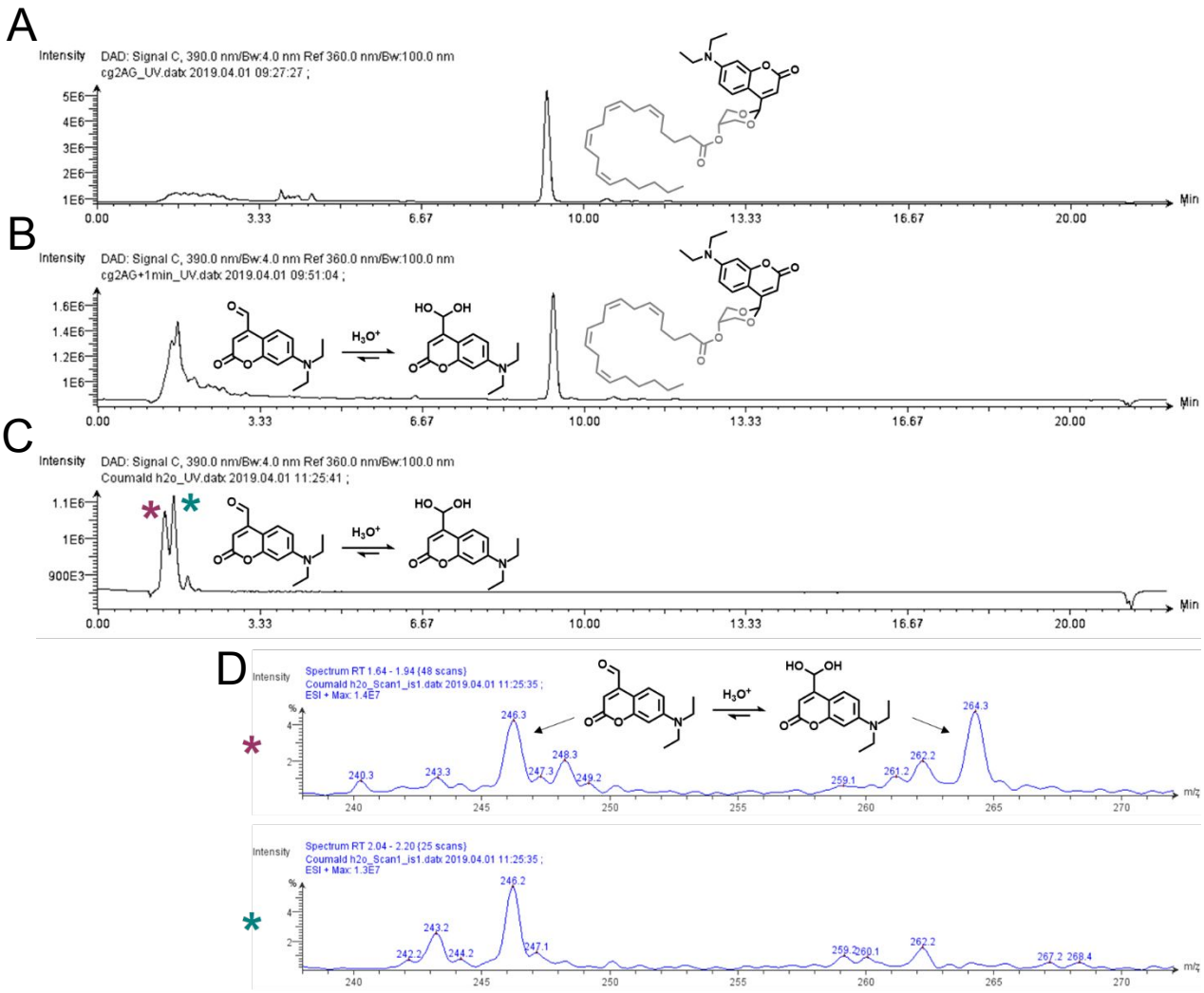


Figure S8: HPLC-MS analysis of a solution containing cg2-AG (10 μM) in H_2O, before (**A**) and after (**B**) 2 min UV light illumination with a 1 kW xenon lamp equipped with a 350 nm highpass filter. The uncaging reaction was monitored by measuring the precence of cg2-AG, 2-AG and intermediate **3** in the absence or presence of light. HRMS (ESI, negative mode) cg2-AG, m/z = 664.38452 $[M+acetate]^-$, 664.38549 calculated for $C_{39}H_{54}NO_8^-$. 2-AG, m/z = 437.29129 $[M+acetate]^-$, 437.29086 calculated for $C_{25}H_{41}O_6^-$ and released coumarin**,** m/z = 260.09238 $[M-H]^-$, 260.09283 calculated for $C_{14}H_{14}NO_4^-$. (**C**) Bar graphs summarizing the relative abundace of each derivatives, before and after UV light illumination.

UV-VIS HPLC analysis.



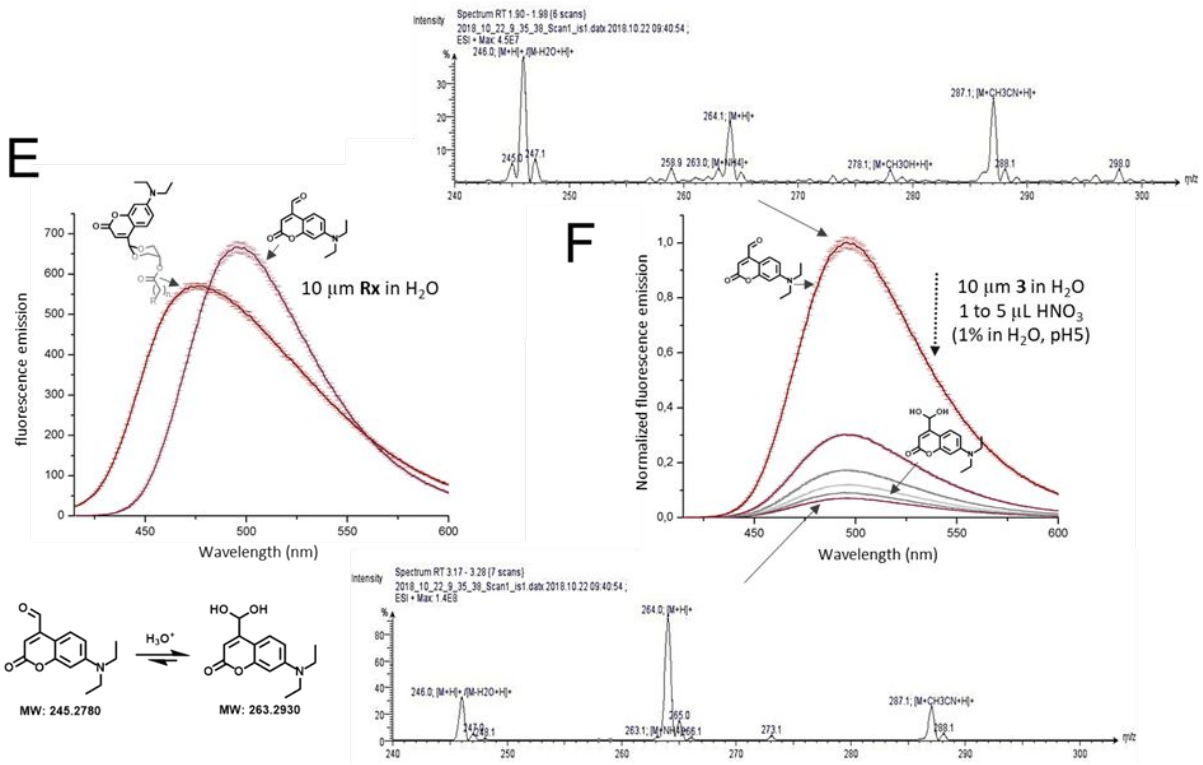


Figure S9: Analysis of a solution containing cg2-AG in H_2O by UV-vis HPLC, before (**A**) and after (**B**) 2 min illumination UV light illumination with a 1 kW xenon lamp equipped with a 350 nm highpass filter. The mobile phase consisted of solvents A (water) and B (acetonitrile), 0.1 % formic acid, gradient: 20 to 100 % of B in 10 min, then back to 20 % within 5 min. **C.** Chromatogram profile of intermediate **3** and mass spectrometry analysis of the two detected peaks (**D**). Intermediate **3**, $[M+H]^+$, m/z = 246.3; hydrate form of **3**, $[M+H]^+$, m/z = 264.3. **(E)** Emission spectra of 10 µM cg2-AG and **3** in H_2O, respectively. **(F)** Emission spectra and MS analysis of aldehyde **3** and its hydration under acidic conditions.

Part 3. Fluorescence investigations.

Absorbance spectra were measured using a UV-1601 UV–VIS spectrophotometer (Shimadzu). The detection range was set to 250–650 nm, the spectral resolution to 0.5nm and the averaging time to 0.1 s. The path length of the cuvette was 1 cm, baseline correction was carried out by subtraction of the background signal of a H_2O sample. All emission spectra were measured using a Cary Eclipse fluorescence spectrophotometer (Agilent). The excitation wavelength was set to 375 nm and emission collection to 400–600 nm with a step size of 3 nm and 0.1 s integration. Spectra were recorded from H_2O solutions and averaged three times.

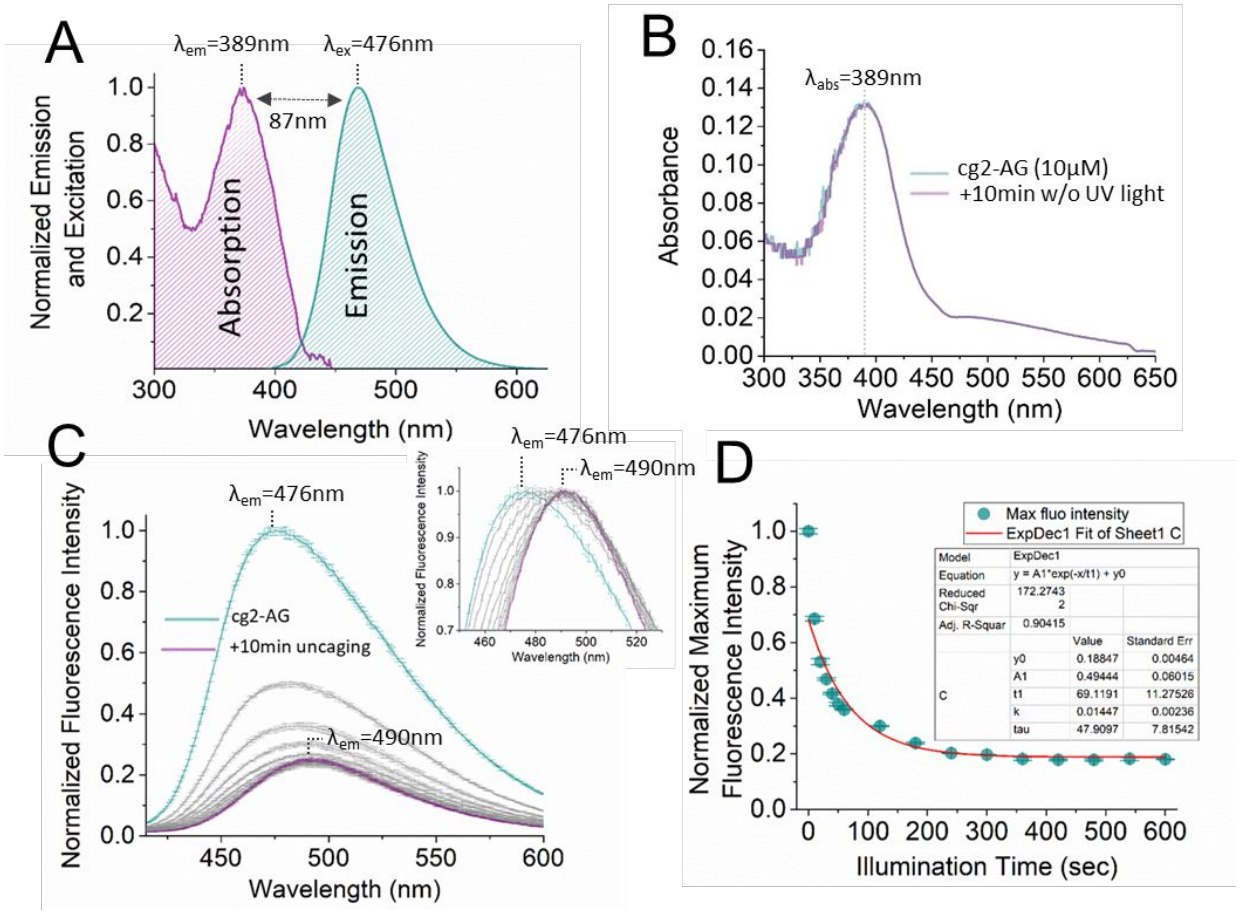


Figure S10: A. Normalized absorption and emission spectra of cg2-AG in H_2O. **B.** UV-VIS absorption spectrum of cg2-AG (10 µM) in H_2O at t_0 and t_{+10min} in absence of light. **C.** Fluorescence emission spectra of cg2-AG (10 µM) in H_2O from t_0 to t_{+10min} of uncaging (with 1min incremental steps) using a 1 kW xenon lamp equipped with a 350 nm highpass filter. Normalized fluorescence emission spectra from 1 to 10 min of uncaging show a red-shifting (from 476 to 490 nm) of the maximum emission wavelength upon the photo-release of 2-AG (top right inlet). **D.** Normalized fluorescence emission intensity changes over the illumination time from 1 to 10 min.

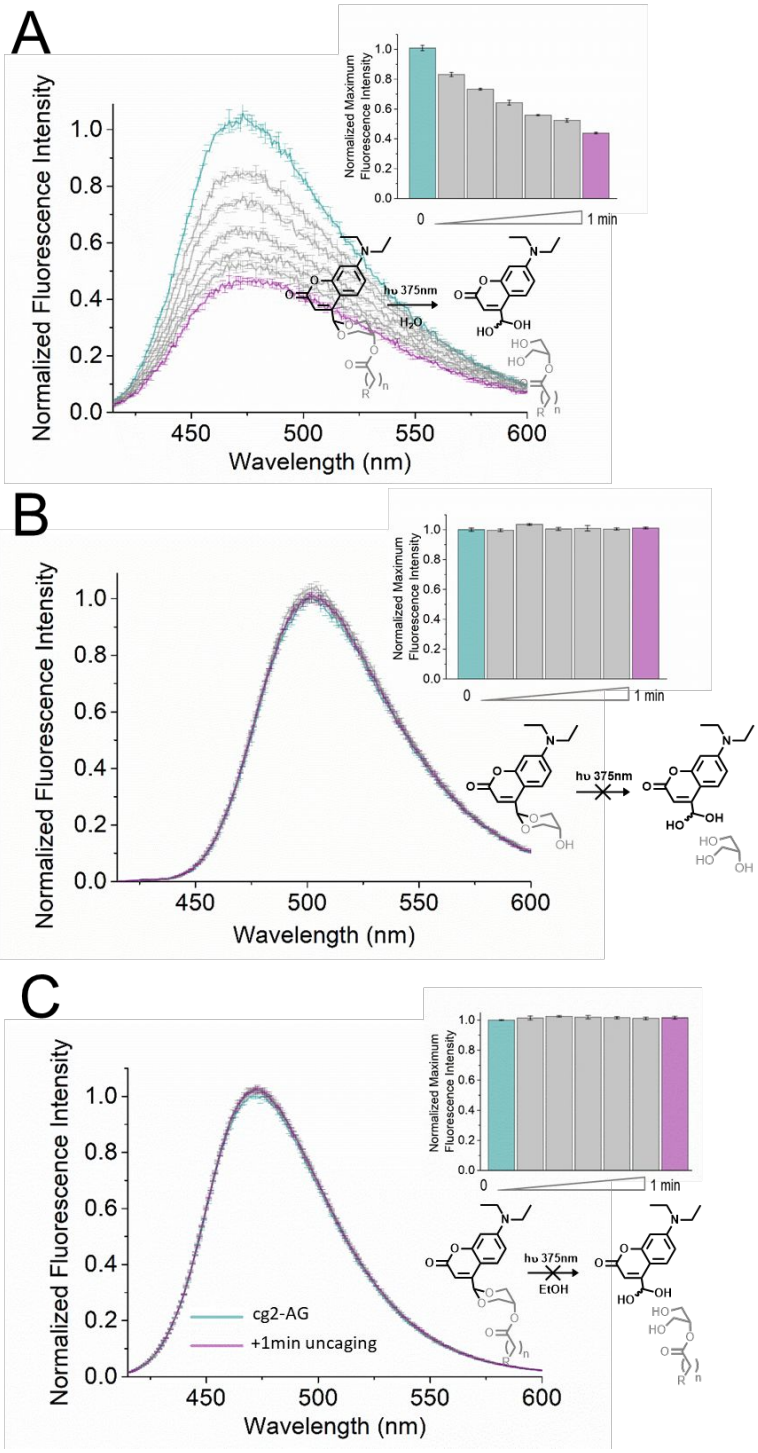


Figure S11: Normalized fluorescence emission spectra from 0 to 1 min illumination with 10 sec incremental steps. Normalized maximum fluorescence intensity changes upon UV irradiation (top right inlets). **A.** cg2-AG (10 μM) in H_2O. **B.** Alcohol **4** (10 μM) in H_2O. **C.** cg2-AG (10 μM) in EtOH.

Part 4. Cell culture and cDNA transfection.

The mouse insulinoma-derived cell line MIN6 used in this study as a model β-cell line was initially developed and kindly provided by Miyazaki et al. [2] Cells were grown in high-glucose DMEM (41965-039, Life Technologies) supplied with 15 % fetal bovine serum (10270098, Life Technologies), and 70 mM β-mercaptoethanol, always added freshly to the cell culture flasks (P07-05100, PAN-Biotech). Cells were seeded in eight-well Lab-Tek microscope dishes 48–64 h (to reach 50–80 % confluence) before imaging. For calcium imaging, MIN6 cells were treated with the cell permeant calcium sensitive dye Fluo-4/AM (5 μM, F14201, Invitrogen). A transfection cocktail of 200 ng EPAC-based cAMP sensor and 200 ng AKAR-4 in 10 μL of Opti-MEM (31985-070, Life Technologies) and 1.5 μL of Lipofectamine 3000 transfection reagent (11668030, Life Technologies) was added to each well of an eight-well Lab-Tek microscope dish loaded with 200 μL high glucose (4.5 g/L) DMEM 24–48 h prior to imaging.

Part 5. Fluorescence microscopy.

Cells were imaged in eight-well Lab-Tek microscope dishes (155411, Thermo Scientific) at 37°C in imaging buffer containing (mM): 20 HEPES, 115 NaCl, 1.2 $CaCl_2$, 1.2 $MgCl_2$, 1.2 K_2HPO_4. MIN6 cells (if not specified otherwise) were imaged in an imaging buffer containing a stimulatory amount of glucose (11 mM). Imaging was performed on a dual scanner confocal microscope Olympus Fluoview 1200, with 20 (air) and/or 63 (oil) objectives. This microscope houses two independent, fully synchronized laser scanners for simultaneous laser stimulation and confocal observation and permits capturing of cellular responses that occur during or immediately following laser stimulation (with a 375 nm wavelength-emitting laser). Microscope settings were adjusted to generate images displaying background fluorescence values slightly larger than zero in order to capture the complete signal stemming from the respective fluorescent dyes or proteins. For imaging, coumarin dyes were excited with 405 nm laser light and emitted light was collected between 425 and 525 nm. CFP/YFP EPAC-based cAMP and AKAR FRET sensors were excited with 440 laser light and emitted light was collected at 465–490 nm and 530–580 nm, respectively.

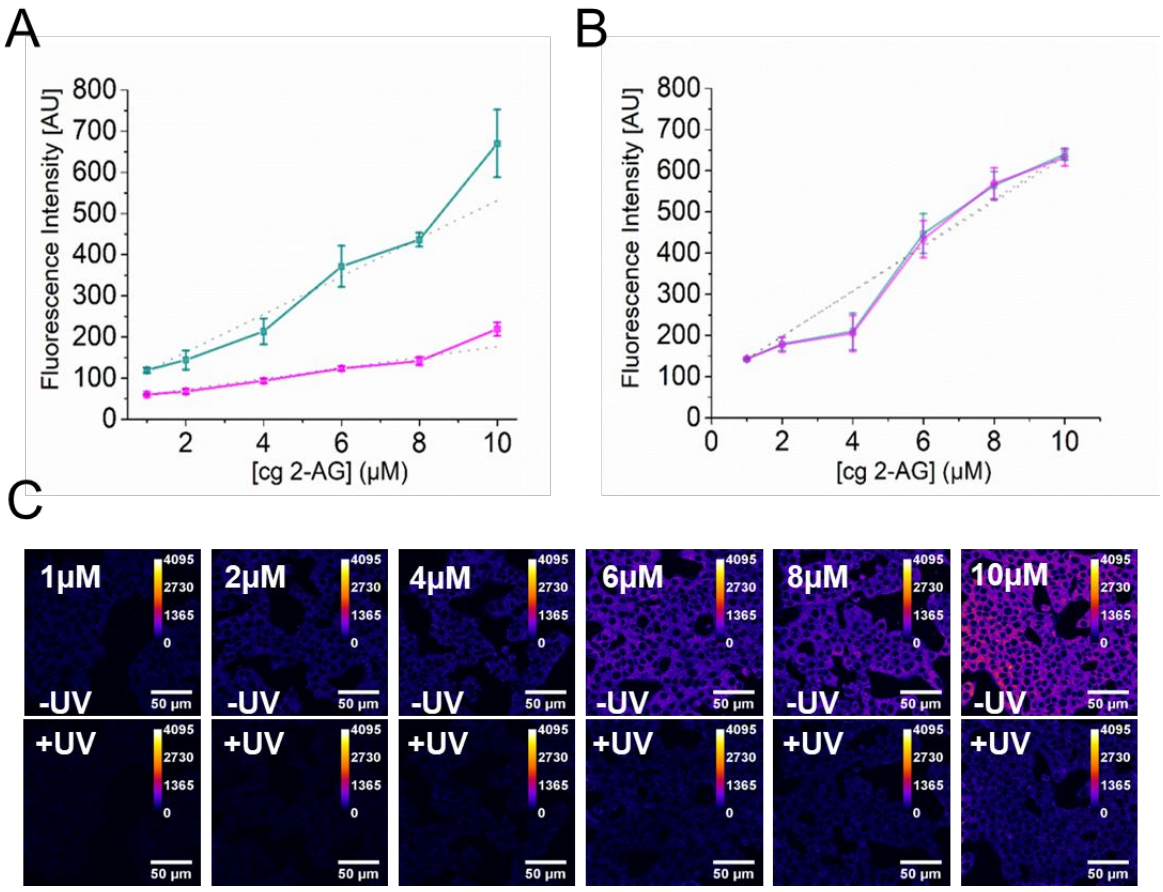
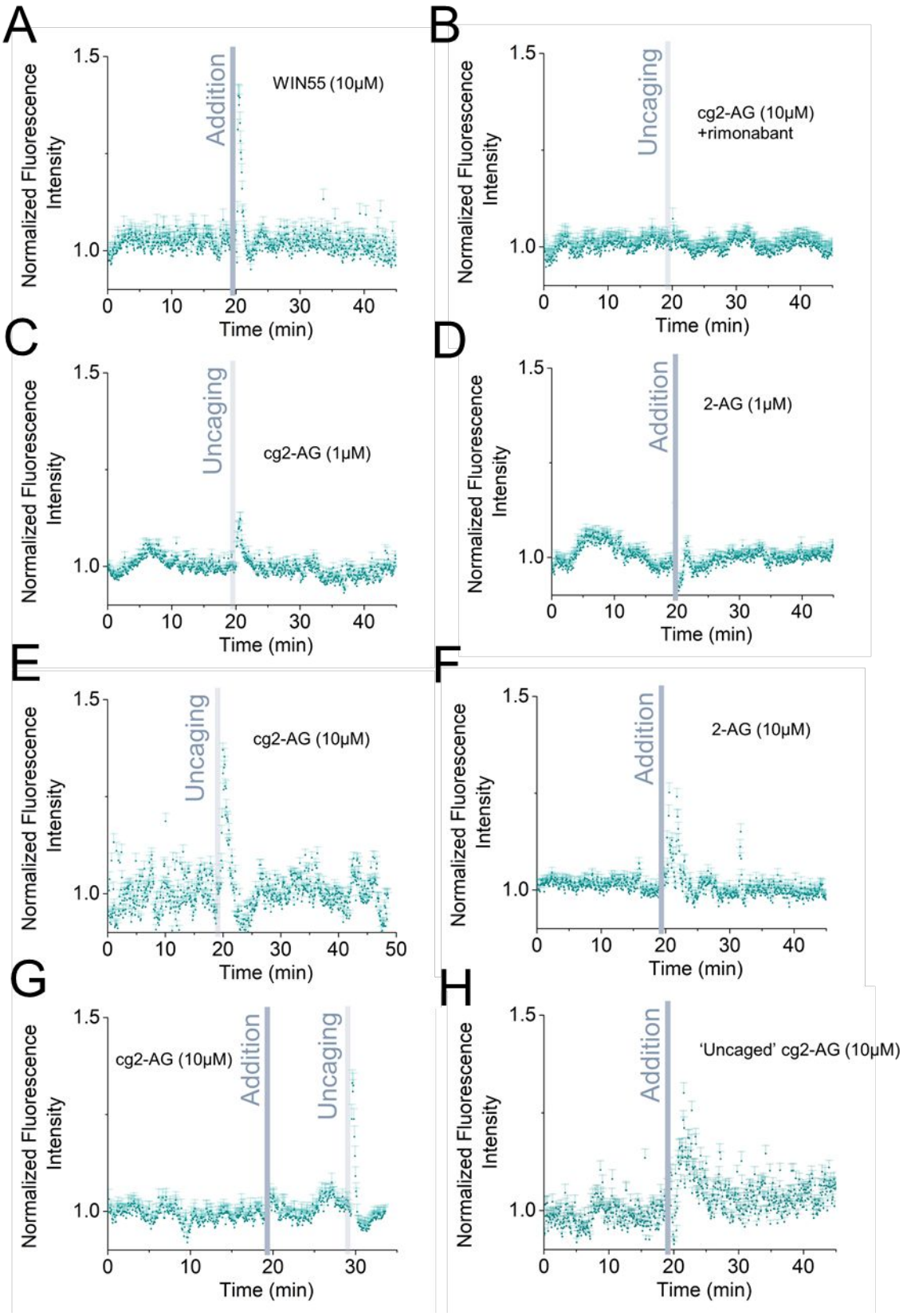


Figure S12: Fluorescence emission intensity of cg2-AG with concentrations varying from 1 μM to 10 μM in live MIN6 cells, before (cyan) and after (pink) uncaging with a 375 nm (**A**) or a 405 nm (**B**) laser through the objective of the confocal microscope. Representative micrographs of MIN6 cells incubated with different cg2-AG concentrations (1μM to 10 μM), before and after uncaging with a 375 nm laser (**C**).


A
Normalized Fluorescence Intensity
1.5
1.0
Addition
WIN55 (10μM)
0
10
20
30
40
Time (min)
B
Normalized Fluorescence Intensity
1.5
1.0
Uncaging
cg2-AG (10μM)
+rimonabant
0
10
20
30
40
Time (min)
C
Normalized Fluorescence Intensity
1.5
1.0
Uncaging
cg2-AG (1μM)
0
10
20
30
40
Time (min)
D
Normalized Fluorescence Intensity
1.5
1.0
Addition
2-AG (1μM)
0
10
20
30
40
Time (min)
E
Normalized Fluorescence Intensity
1.5
1.0
Uncaging
cg2-AG (10μM)
0
10
20
30
40
50
Time (min)
F
Normalized Fluorescence Intensity
1.5
1.0
Addition
2-AG (10μM)
0
10
20
30
40
Time (min)
G
Normalized Fluorescence Intensity
1.5
1.0
cg2-AG (10μM)
Addition
Uncaging
0
10
20
30
Time (min)
H
Normalized Fluorescence Intensity
1.5
1.0
Addition
'Uncaged' cg2-AG (10μM)
0
10
20
30
40
Time (min)

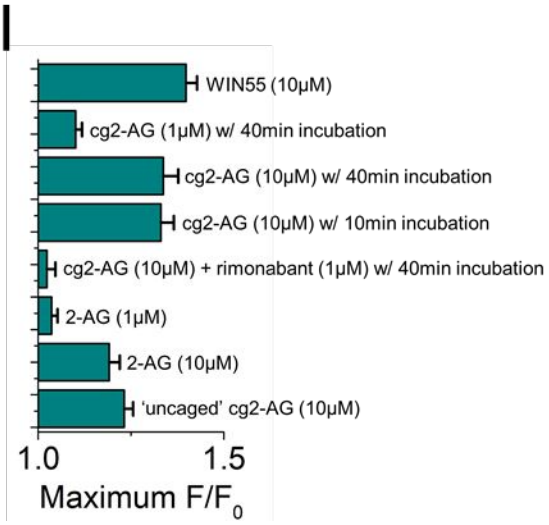


Figure S13: Normalized fluorescence intensity increase F/F_0 of live MIN6 pre-treated with the cell permeant calcium dye Fluo4-AM (5 μM) in presence of (**A**) the CB1 full agonist WIN55 ([WIN55 = 10 μM, n = 84); (**B**) cg2-AG incubated 40 minutes before uncaging ([cg2-AG] = 1 μM, n = 183), (**C**) cg2-AG incubated 40 minutes before uncaging ([cg2-AG] = 10 μM, n = 136), (**D**) cg2-AG incubated 10 minutes before uncaging ([cg2-AG] = 10 μM, n = 81), (**E**) cg2-AG in presence of the CB1 antagonist rimonabant (1μM; [cg2-AG] = 10 μM, n = 79), (**F**) 2-AG ([2-AG] = 1 μM, n = 148), (**G**) 2-AG ([2-AG] = 10 μM, n = 150) and subjected to flash photolysis (λ_{ex} = 488 nm, $\lambda_{uncaging}$ = 375 nm) or (**H**) cg2-AG ([2-AG] = 10 μM, n = 98) subjected to photolysis (5 min in imaging buffer (IB) under a 1 kW xenon lamp equipped with a 350 nm highpass filter) before addition onto the cells. The baseline noise is due to frequently occurring oscillations in calcium levels. Recapitulative bar graph comparing the maximum increase of fluorescence F/F_0 for each conditions (**I**).

Part 6. Stability of cg2-AG in MIN6 cells

After removing the growth medium (1.5 ml/dish), MIN6 cells were washed with DPBS (2x 2 ml) and incubated with IB + glucose (2 ml, 11 mM) for 30 min. cg2-AG (10 μM) was added and the cells were incubated at 37°C for 30 min. Cells were washed with DPBS (1x 2 ml) and new IB + glucose was added (2 ml, 11mM) before UV irradiation (2 min with 350 nm highpass band filter). The supernatant was removed and 100 μl of IB was added. After scraping the cells, 120 μl of the preparation was harvested and directly frozen in liquid nitrogen. Samples were further processed through a methanol/chloroform lipid extraction and lipid extracts were analyzed by HPLC-MS.

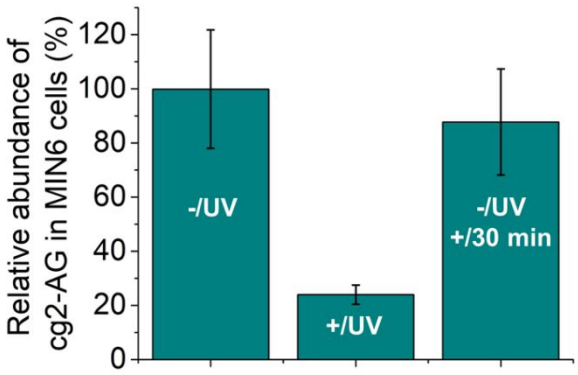


Figure S14: Relative abundance of cg2-AG in MIN6 cells pre-incubated (10 μM) for 30 min in absence or presence of UV irradiation, measured directly after pre-incubation or after another 30 min at 37°C. The analysis shows that UV light leads to a drop in cg2-AG levels and very little metabolism of the caged 2-AG within 30 min.

Part 7. Electrophysiology

Whole-cell, patch recordings were performed in voltage clamp and current clamp using an Olympus BX51W1 upright microscope equipped with video-enhanced, infrared-differential interference contrast (IR-DIC) and an Exfo X-Cite 120 Series fluorescence light source. Electrodes were fabricated from borosilicate glass (1.5 mm outer diameter; World Precision Instruments, Sarasota, FL) and filled with a normal internal solution (in mM): 128 potassium gluconate, 10 NaCl, 1 $MgCl_2$, 11 EGTA, 10 HEPES, 3 ATP, and 0.25 GTP (pH was adjusted to 7.3–7.4 with 1N KOH, 290–300 mOsm). Pipette resistances ranged from 3–5 MΩ. In whole cell configuration, access resistance was less than 20 MΩ. Membrane currents underwent analog-to digital conversion via a Digidata 1322A interface coupled to pClamp 9.2 (Axon Instruments). Low-pass filtering of the currents was conducted at a frequency of 2 kHz. The liquid junction potential was -10 mV and was corrected for in subsequent data analysis. To display reversal potential and rectification characteristics of the photo-stimulation or ligand-activated currents, I–V plots constructed by voltage ramps from 100 to -100 mV were applied at 500 ms or 2 s intervals from a holding potential of -60 mV. For photo-activation, a light-induced response was evoked using a light-emitting diode (LED) 375 nm blue light source controlled by a variable 2A driver (ThorLabs, Newton, NJ) with the light path delivered directly through an Olympus 40 × water-immersion lens.

Unpaired t test	
P value	0.0447
P value summary	*
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=2.441 df=7
How big is the difference?	
Mean ± SEM of column A	13.92 ± 3.878, n=5
Mean ± SEM of column B	3.150 ± 0.3948, n=4

Unpaired t test	
P value	0.1041
P value summary	ns
Significantly different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=1.808 df=9
How big is the difference?	
Mean ± SEM of column A	13.92 ± 3.878, n=5
Mean ± SEM of column C	32.67 ± 8.828, n=6

Part 8. References

1. Walter, A.M., et al., *Phosphatidylinositol 4,5-bisphosphate optical uncaging potentiates exocytosis.* Elife, 2017. **6**, e30203.
2. Blankman, J.L., G.M. Simon, and B.F. Cravatt, *A comprehensive profile of brain enzymes that hydrolyze the endocannabinoid 2-arachidonoylglycerol.* Chem Biol, 2007. **14**(12): p. 1347-56.