SUPPORTING INFORMATION

“Close-to-Release”: Spontaneous Bioorthogonal Uncaging Resulting from Ring-Closing Metathesis

Valerio Sabatino, Johannes G. Rebelein and Thomas R. Ward*

Department of Chemistry, University of Basel, Switzerland

*Corresponding Author: Thomas.ward@unibas.ch
# TABLE OF CONTENTS

S1.-GENERAL INFORMATION ........................................................................................................... 4

S2.-SYNTHESIS OF SUBSTRATES (1, 3a-g, 4a-c, 5a-d, 6) ................................................................ 6
  S2.1.-Synthetic scheme for the preparation of 1-(2-allylphenyl)prop-2-en-1-ol (1) ................. 6
  S2.2.-General procedure for the preparation of esters 3a-g ......................................................... 7
  S2.3.-General procedure for the preparation of ethers 4a-b ......................................................... 8
  S2.4.-General procedure for the preparation of carbamates 5a-d ............................................. 8
  S2.5.-Synthesis of carbonate 6 ...................................................................................................... 9

S3.-CHARACTERIZATION OF SUBSTRATES (1, 3a-g, 4a-c, 5a-d, 6) ......................................... 10
  S3.1.-Characterization of esters 3a-g ............................................................................................ 10
  S3.2.-Characterization of ethers 4a-b .......................................................................................... 13
  S3.3.-Characterization of carbamates 5a-d .................................................................................. 14

S4.-1H NMR STUDIES OF THE RU-TRIGGERED UNCAGING REACTION OF ESTERS ............. 15
  S4.1.-Stability of the ester 3c ........................................................................................................ 15
  S4.2.-NMR studies of the uncaging reaction of ester 3c .............................................................. 18

S5.-QUANTITATIVE GC-MS ANALYSIS ......................................................................................... 20
  S5.1.-General procedure .............................................................................................................. 20
  S5.2.-Calibration curve of naphthalene 2 for GC-MS quantitative analysis .............................. 20
  S5.3.-Catalysts screening with 1-(2-allylphenyl)prop-2-en-1-ol (1) ........................................... 21
  S5.4.-Quantitative GC-MS analysis of esters 3, ethers 4, carbamates 5 and carbonates 6 ..... 22
  S5.5.-Monitoring the uncaging reaction of ester 3d over time .................................................. 23

S6.-FLUORESCENCE EXPERIMENTS OF FLUOROGENIC SUBSTRATES 4a AND 5c .............. 23
  S6.1.-RCM-triggered uncaging of fluorogenic ether 4a .............................................................. 23
  S6.2.-Kinetic studies of the uncaging reaction of substrate 4a .................................................... 25
  S6.3.-RCM-triggered uncaging of substrate 5c ........................................................................... 26

S7.-RCM-TRIGGERED 1,4-ELIMINATION IN COMPLEX BIOLOGICAL MEDIA ......................... 27
  S7.1.-General procedure .............................................................................................................. 28
  S7.2.-RCM of ether 4a in DMEM ................................................................................................ 28
  S7.3.-RCM of ether 4a in E.coli cell lysates ................................................................................. 29
  S7.4.-RCM of ether 4a in the presence of E. coli cells ................................................................. 30

S8.-RCM-TRIGGERED UNCAGING IN THE PERIPLASM OF E.COLI ...................................... 33

S9.-RCM-TRIGGERED UNCAGING OF UMBELLIFERONE IN THE PRESENCE OF MAMMALIAN CELLS ............................................................................................................ 36
S9.1.- General procedure ................................................................. 36
S9.2.- Cell Viability Determination .................................................. 39
S10.- NMR AND HR-MS SPECTRA .................................................. 41
S11.- STABILITY OF ETHERS, CARBAMATES AND CARBONATES IN THE
REACTION MEDIA ........................................................................ 80
   S11.1.- General procedure for the evaluation of the stability of model substrates 4b,
           5c and 6 .................................................................................. 80
   S11.2.- 1H NMR spectra of the substrates 4b, 5c and 6 after 1-hour and 24-hours 80
S12.- REFERENCES ......................................................................... 84
S1.-GENERAL INFORMATION

Procedures for the synthesis of precursors were performed under an atmosphere of dry nitrogen using vacuum-line and standard Schlenk techniques. Dry solvents were directly purchased from Sigma Aldrich or Acros Organics and used without further purification. Water used in the catalytic reactions was purified by Milli-Q Advantage system.

Chemicals were purchased from Sigma Aldrich, Acros Organics, Alfa Aesar, Fluorochem, Apeiron synthesis and used without further purification. 2-allylbenzaldehyde was synthesized according to the literature. The $^1$H and $^{13}$C NMR data were in agreement with the reported values.

All catalytic reactions were carried out with non-degassed solvents under air. Reaction mixtures were stirred using Teflon-coated magnetic stir bars. The abbreviation “rt” refers to reactions carried out approximately at 23 °C. Temperature was maintained using Thermowatch-controlled heating blocks. Thin-layer chromatography (TLC) was performed on silica gel plates and components were visualized by observation under UV light and / or by treating the plates with potassium permanganate (KMnO$_4$) followed by heating. Flash chromatography was carried out on silica gel. Drying was achieved with anhydrous Na$_2$SO$_4$.

Concentration refers to the removal of volatile solvents via distillation using a rotary evaporator Büchi R-300HL equipped with a thermostated bath B-300, a vacuum regulator CVC-3000, followed by residual solvent removal under high vacuum.

$^1$H NMR (500 MHz) and $^{13}$C NMR (126 MHz) spectra were recorded at room temperature on a Bruker 500 MHz spectrometer. Data are represented as follows:
chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad signal, bs = broad singlet, dd = doublet doublets, dt = doublet triplets, dq = doublet quartets, td = triplet doublets, ddd = doublet of doublet of doublets, ddt = doublet of doublet of triplets, dttd = doublet of triplet of doublets, dddd = doublet of doublet of doublet of doublets), coupling constants in Hertz (Hz). The chemical shifts for protons (δ) are reported in parts per million downfield from tetramethylsilane and are referenced to residual protium in the NMR solvent (CHCl₃ δ = 7.26). Chemical shifts for carbon are reported in parts per million downfield from tetramethylsilane and are referenced to the carbon resonances of the solvent (CDCl₃ δ = 77.0). NMR spectra were analyzed using MestreNova© NMR data processing software (www.mestrelab.com).

Quantitative GC-MS analysis was performed on a Shimadzu GCMS-QP2010S equipped with Agilent HP1-1MS (length: 30 m; Diameter: 0.25 mm; Film: 0.25 µM). GC column flow 2.05 mL/min Helium at linear velocity mode. GC inlet Temperature 250 °C, Split Ratio 80:1 – 99:1, oven Temperature 50 °C, hold for 1.0 min, 20 °C/min to 230 °C, hold 1 min. Runtime is 10.0 min. MS parameters: ion source Temperature 230 °C, interface Temperature 280 °C, Scan range m/z 50-500, scan time 2.5-10 min, Single Ion Mass (ch1 m/z 158.00, ch2 m/z 128). Quantitative parameters (target, m/z 128, retention time 5.700 min; internal standard (1-methoxynaphthalene), m/z 158, retention time 7.550 min). Injection volume: 1 µL.

High resolution mass spectra (HRMS) were acquired using electrospray (ESI) and were recorded at the analytical facility of the Chemistry department of the University of Basel.
Fluorescence assays were recorded on Tecan fluorimeter Infinite M1000Pro. Samples were prepared on a 96-well plate (ThermoFischer Scientific Nunclon 96 Flat Black). Measurements settings: increment 1.0 nm, averaging time 0.1 s, excitation slit width 5.0 nm, emission slit width 5.0 nm, PMT voltage 620 V. Excitation 325 nm, Emission 450 nm Bandwidth interval 5.0 nm, Settle time 0.1 s. Kinetic experiments settings: Temperature 37 °C, Kinetic cycle 14-25 (kinetic interval: 5.00 min).

S2. SYNTHESES OF SUBSTRATES (1, 3a-g, 4a-c, 5a-d, 6)

S2.1. Synthetic scheme for the preparation of 1-(2-allylphenyl)prop-2-en-1-ol (1)

\[
\begin{align*}
\text{B(OH)}_2 & \quad + \quad \text{Br-} & \quad \xrightarrow{\text{Pd(PPh}_3\text{)}_2\text{Cl}_2, \text{aq. Na}_2\text{CO}_3, \text{THF, reflux, 16 h}} & \quad \text{2-allylbenzaldehyde} \\
\text{Ph} & \quad \text{CH} \quad & \quad \text{Br} & \quad \text{MgBr} & \quad \text{THF, rt, 4h} & \quad \text{1}
\end{align*}
\]

Step 1: synthesis of 2-allylbenzaldehyde

A solution of 2-formylphenyl boronic acid (190.0 mg, 1.0 mmol, 1.0 eq.) and allyl bromide (0.1 mL, 1.2 eq.) in dry THF was added to a round-bottom flask at room temperature. Pd(PPh\(_3\))\(_2\)Cl\(_2\) (18.0 mg, 0.025 mmol) and aqueous Na\(_2\)CO\(_3\) (1 M, 2.0 mmol, 2.0 eq.) were added and the resulting mixture refluxed overnight. The reaction mixture was quenched with H\(_2\)O and extracted with dichloromethane (DCM) three times. The combined organic layers were washed with H\(_2\)O, dried over anhydrous Na\(_2\)SO\(_4\) and concentrated under reduced pressure. The residues were purified by column chromatography on silica gel (cyclohexane:ethyl acetate 30:1) to afford 2-allylbenzaldehyde (60% yield) as a pale yellow oil. The data reported are consistent
with reported data. $^1$H NMR (500 MHz, Chloroform-$d$) $\delta$ 10.19 (s, 1H), 7.78 (dd, $J = 7.7, 1.5$ Hz, 1H), 7.46 (td, $J = 7.5, 1.5$ Hz, 1H), 7.33 (td, $J = 7.5, 1.2$ Hz, 1H), 7.23 (dd, $J = 7.6, 1.1$ Hz, 1H), 5.97 (ddt, $J = 17.2, 10.1, 6.2$ Hz, 1H), 5.02 (dq, $J = 10.1, 1.5$ Hz, 1H), 4.92 (dq, $J = 17.1, 1.7$ Hz, 1H), 3.75 (dt, $J = 6.3, 1.7$ Hz, 2H). $^{13}$C NMR (126 MHz, Chloroform-$d$) $\delta$ 192.34, 142.27, 136.94, 133.97, 133.87, 131.60, 131.08, 126.92, 116.42, 36.54, 26.93.

Step 2:
A solution of 2-allybenzaldehyde (3.0 g, 20.5 mmol, 1.0 eq) in dry THF (10 mL) was added to a round bottom flask. Vinyl magnesium bromide (1.0 mM solution in THF, 24.6 mmol, 1.2 eq.) was added dropwise to the solution at room temperature. The resulting mixture was stirred for 4 hours at room temperature. At reaction completion, the reaction was quenched with saturated NH$_4$Cl and extracted with ethyl acetate (EtOAc, 3 x 50 mL). The organic phase was dried over anhydrous Na$_2$SO$_4$ and concentrated under reduced pressure. The crude mixture was purified by silica gel column chromatography (9:1 cyclohexane:ethyl acetate), to give (1) as a pale yellow oil (2.5 g, 73% yield). $^1$H NMR (500 MHz, Chloroform-$d$) $\delta$ 7.41 – 7.38 (m, 1H), 7.20 – 7.17 (m, 3H), 7.13 – 7.09 (m, 1H), 6.05 – 5.87 (m, 2H), 5.40 (dt, $J = 5.3, 1.6$ Hz, 1H), 5.27 (dt, $J = 17.1, 1.5$ Hz, 1H), 5.15 (dt, $J = 10.4, 1.5$ Hz, 1H), 5.02 (dq, $J = 10.1, 1.6$ Hz, 1H), 4.93 (dq, $J = 17.1, 1.8$ Hz, 1H), 3.43 (dd, $J = 6.3, 1.7$ Hz, 2H). $^{13}$C NMR (126 MHz, Chloroform-$d$) $\delta$ 140.44, 139.73, 137.45, 137.12, 130.06, 127.94, 126.89, 126.65, 116.01, 115.02, 71.36, 36.75. HRMS (ESI$^+$): $m/z$ calcd for C$_{12}$H$_{14}$O, [M+H]$^+$, 175.10; found 175.09, C$_{12}$H$_{14}$O, [M+H], 197.09 C$_{12}$H$_{14}$ONa [M+H+Na]$^+$.

S2.2.-General procedure for the preparation of esters 3a-g.
In an oven-dried 25 mL flask, a solution of DCC (66.0 mg, 1.2 eq.), DMAP (2.5 mg, 0.1 eq.) and carboxylic acid (1.0 eq.) in dry DCM (5 mL) was stirred at room temperature. A solution of alcohol (100.0 mg, 1.0 eq.) in dry DCM was added dropwise at room temperature, and the resulting mixture was stirred for 16 hours. The reaction was quenched with water (10 mL) and extracted with ethyl acetate (3x15 mL). The organic layer was dried over Na$_2$SO$_4$ and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography.

S2.3.-General procedure for the preparation of ethers 4a-b.

A mixture of 1 (100.0 mg, 1.0 eq.), triphenylphosphine (113.0 mg, 1.5 eq.) and alcohol (50-100.0 mg, 1.0 eq.) in dry THF were added to an oven-dried round-bottom flask at room temperature. A solution of DIAD (87.1 mg, 1.5 eq.) in dry THF was added dropwise at room temperature. The mixture was concentrated under reduced pressure and purified by column chromatography.

S2.4.-General procedure for the preparation of carbamates 5a-d.
To a solution of generic amine (50-100 mg, 1.0 eq.) in dry CH\textsubscript{2}Cl\textsubscript{2} (2 mL), triphosgene (49.1 mg, 0.15 mmol, 0.5 eq.) and NaHCO\textsubscript{3} (25.2 mg, 0.3 mmol, 3.0 eq.) were added. The mixture was stirred until reaction completion (monitored by TLC) and the volatiles were evaporated under reduced pressure. The crude material was dissolved in anhydrous DCM and added dropwise to a solution of allylic-alcohol 1 (50 mg, 1.0 eq.) and triethylamine (0.05 mL, 1.1 eq.) in anhydrous DCM. After reaction completion, the mixture was concentrated under reduced pressure and the resulting crude was purified by silica gel column chromatography.

S2.5.-Synthesis of carbonate 6

In a 25 mL round-bottom flask charged with alcohol (100.0 mg, 1.0 eq.) and dry DCM, triethylamine (0.1 mL, 1.24 eq.) was added, and the mixture cooled to 0 °C. After 10 minutes, a mixture of \textit{p}-nitrophenyl chloroformate (116 mg, 1.0 eq.) in dry DCM was added dropwise to the mixture and let to warm up to rt. Upon reaction completion (monitored by TLC), the mixture was concentrated under reduced pressure and purified by silica gel column chromatography (9:1 cyclohexane: ethyl acetate), to yield the desired carbonate 6 as a pale yellow oil (50 mg, 26%). \textsuperscript{1}H NMR (500 MHz,
1. (2-allylphenyl)allyl nicotinate (3a)

Prepared according to the general procedure for esters (S2.2). Purified by silica gel column chromatography (9:1 cyclohexane:ethyl acetate). Pale yellow oil (63% yield 3a) ¹H NMR (500 MHz, Chloroform-d) δ 9.28 (dd, J = 2.2, 0.9 Hz, 1H), 8.78 (dd, J = 4.8, 1.8 Hz, 1H), 8.32 (dt, J = 8.0, 2.0 Hz, 1H), 7.53 – 7.48 (m, 1H), 7.39 (dd, J = 8.0, 4.9, 0.9 Hz, 1H), 7.30 – 7.26 (m, 2H), 7.24 – 7.20 (m, 1H), 7.67 (dt, J = 5.5, 1.6 Hz, 1H), 6.12 (ddd, J = 17.1, 10.6, 5.4 Hz, 1H), 6.05 – 5.95 (m, 1H), 5.35 – 5.28 (m, 2H), 5.11 – 4.99 (m, 2H), 3.67 – 3.50 (m, 2H). ¹³C NMR (126 MHz, Chloroform-d) δ 164.21, 153.53, 151.02, 137.67, 137.14, 136.75, 136.52, 135.75, 130.14, 128.56, 127.64, 126.86, 126.15, 123.29, 117.32, 116.34, 73.93, 36.92. HRMS (ESI⁺): m/z calcd for C₁₈H₁₇NO₂, [M+H]⁺, 280.13; found 280.13, C₁₈H₁₇NO₂H [M+H]⁺.

1. (2-allylphenyl)allyl 2-chlorobenzoate (3b)

Prepared according to general procedure for esters (S2.2). Purified by silica gel column chromatography (9:1 cyclohexane:ethyl acetate). Pale yellow oil (72% yield 3b) ¹H NMR (500 MHz, Chloroform-d) δ 7.86 (dd, J = 7.8, 1.7 Hz, 1H), 7.54 – 7.50 (m, 1H), 7.47 – 7.39 (m, 2H), 7.35 – 7.26 (m, 3H), 7.24 – 7.19 (m, 1H), 6.75 (dt, J = 5.5, 1.6 Hz, 1H), 6.12 (ddd, J = 17.1, 10.5, 5.4 Hz, 1H), 6.01 (ddt, J = 16.7, 10.1, 6.3 Hz, 1H), 5.36 – 5.27 (m, 2H), 5.12 – 5.00 (m, 2H), 3.69 – 3.49 (m, 2H). ¹³C NMR (126 MHz, Chloroform-d) δ 164.53, 137.68, 136.83, 136.62, 135.86, 133.92, 132.61, 131.59, 131.15, 130.03, 129.98, 128.41, 127.83, 126.78,
126.57, 117.31, 116.32, 74.12, 36.97. HRMS (ESI+): m/z calcd for C$_{19}$H$_{17}$ClO$_2$, [M+H]$^+$, 313.09; found 313.09, C$_{19}$H$_{17}$ClO$_2$H [M+H]$^+$. 

1-(2-allylphenyl)allyl 3-bromo-4-nitrobenzoate (3c)

![Chemical structure of 3c]

Prepared according to general procedure for esters (S2.2). Purified by silica gel column chromatography (4:1 cyclohexane: ethyl acetate). Light yellow oil (75% yield 3c) $^1$H NMR (500 MHz, Chloroform-d) δ 8.29 – 8.21 (m, 2H), 8.20 – 8.11 (m, 2H), 7.55 – 7.47 (m, 1H), 7.31 – 7.27 (m, 2H), 7.25 – 7.21 (m, 1H), 6.76 (dt, J = 5.4, 1.5 Hz, 1H), 6.22 – 6.08 (m, 1H), 6.00 (ddt, J = 16.6, 10.1, 6.3 Hz, 1H), 5.36 – 5.27 (m, 2H), 5.14 – 4.97 (m, 2H), 3.71 – 3.44 (m, 2H). $^{13}$C NMR (126 MHz, Chloroform-d) δ 164.05, 137.72, 136.73, 136.41, 136.10, 135.64, 133.04, 130.21, 130.19, 130.05, 130.03, 128.63, 127.61, 126.89, 117.47, 116.38, 74.33, 36.94. HRMS (ESI+): m/z calcd for C$_{19}$H$_{16}$BrN$_2$O$_3$, [M+H]$^+$, 402.03; found 402.02, C$_{19}$H$_{16}$BrN$_2$O$_3$, [M+H]$^+$. 402.02, C$_{19}$H$_{16}$BrN$_2$O$_3$Na [M+H+Na]$^+$. 

1-(2-allylphenyl)allyl 4-(2,2,2-trifluoroacetyl)benzoate (3d)

![Chemical structure of 3d]

Prepared according to the general procedure for esters (S2.2). Purified by silica gel column chromatography (9:1 cyclohexane: ethyl acetate). Light yellow oil (60% yield 3d) $^1$H NMR (500 MHz, Chloroform-d) δ 8.29 – 8.21 (m, 2H), 8.20 – 8.11 (m, 2H), 7.55 – 7.47 (m, 1H), 7.31 – 7.27 (m, 2H), 7.25 – 7.21 (m, 1H), 6.76 (dt, J = 5.4, 1.5 Hz, 1H), 6.22 – 6.08 (m, 1H), 6.00 (ddt, J = 16.6, 10.1, 6.3 Hz, 1H), 5.36 – 5.27 (m, 2H), 5.14 – 4.97 (m, 2H), 3.71 – 3.44 (m, 2H). $^{13}$C NMR (126 MHz, Chloroform-d) δ 164.05, 137.72, 136.73, 136.41, 136.10, 135.64, 133.04, 130.21, 130.19, 130.05, 130.03, 128.63, 127.61, 126.89, 117.47, 116.38, 74.33, 36.94. HRMS (ESI+): m/z calcd for C$_{21}$H$_{17}$F$_3$O$_3$, [M+H]$^+$, 375.11; found 375.10, C$_{21}$H$_{17}$F$_3$O$_3$, [M+H]$^+$. 375.10, C$_{21}$H$_{17}$F$_3$O$_3$Na [M+H+Na]$^+$. 

1-(2-allylphenyl)allyl 2-propylpentanoate (3e)

![Chemical structure of 3e]

Prepared according to the general procedure for esters (S2.2). Purified by silica gel column chromatography (9:1 cyclohexane: ethyl acetate). Light yellow oil (80% yield 3e) $^1$H NMR (500 MHz, Chloroform-d) δ 7.42 – 7.35 (m, 1H), 7.25 – 7.21 (m, 2H), 7.20 – 7.16 (m, 1H), 6.49 (dt, J = 5.4, 1.6 Hz, 1H), 6.03 – 5.94 (m, 2H), 5.24 – 5.18 (m, 2H), 5.08 (dq, J = 10.1, 1.6 Hz, 1H), 5.02 (dq, J = 17.0, 1.7 Hz, 1H), 3.63 – 3.43 (m, 2H), 2.42 (tt, J = 8.9, 5.3 Hz, 1H), 1.67 – 1.51 (m, 2H), 1.48 – 1.36 (m, 2H), 1.31 – 1.16 (m,
12

H), 0.86 (dt, \( J = 20.7, 7.3 \) Hz, 6H). \(^{13}\)C NMR (126 MHz, Chloroform-\( d_2 \)) \( \delta \) 175.38, 137.51, 137.19, 136.92, 136.45, 128.10, 127.51, 126.54, 116.64, 116.16, 72.35, 45.32, 36.85, 34.60, 34.57, 20.59, 20.46, 14.01, 13.97. HRMS (ESI\(^+\)): \( m/z \) calcd for C\(_{20}\)H\(_{28}\)O\(_2\), [M+H]\(^+\), 301.21; found 301.20, C\(_{20}\)H\(_{28}\)O\(_2\), [M+H]\(^+\), 323.20, C\(_{20}\)H\(_{28}\)O\(_2\)Na [M+H+Na]\(^+\).

1-(2-allylphenyl)allyl 3-(N-(4-methyl-2-nitro-5-(pyridin-3-yl)phenyl)sulfamoyl)benzoate (3f)

Prepared according to the general procedure for esters (S2.2). Purified by silica gel column chromatography (9:1 cyclohexane: ethyl acetate). Yellow oil (10% yield 3f) \(^1\)H NMR (500 MHz, Chloroform-\( d_2 \)) \( \delta \) 9.73 (s, 1H), 8.62 (dd, \( J = 4.9, 1.7 \) Hz, 1H), 8.46 (d, \( J = 2.3 \) Hz, 1H), 8.41 (t, \( J = 1.8 \) Hz, 1H), 8.20 (dt, \( J = 7.8, 1.4 \) Hz, 1H), 7.96 (dt, \( J = 7.4, 1.4 \) Hz, 2H), 7.66 (s, 1H), 7.55 – 7.50 (m, 2H), 7.39 – 7.31 (m, 2H), 7.22 – 7.19 (m, 2H), 7.14 (dd, \( J = 7.1, 2.0 \) Hz, 1H), 6.65 (dt, \( J = 5.5, 1.5 \) Hz, 1H), 6.03 (ddd, \( J = 17.1, 10.5, 5.5 \) Hz, 1H), 5.91 (ddt, \( J = 16.6, 10.1, 6.3 \) Hz, 1H), 5.25 – 5.15 (m, 2H), 5.01 – 4.89 (m, 2H), 3.58 – 3.40 (m, 2H), 2.17 (s, 3H). \(^{13}\)C NMR (126 MHz, Chloroform-\( d_2 \)) \( \delta \) 163.44, 149.50, 148.83, 146.18, 139.37, 137.67, 136.72, 136.51, 136.35, 136.26, 135.60, 134.63, 132.77, 131.84, 131.17, 131.08, 130.16, 129.82, 128.63, 128.33, 127.72, 127.55, 126.94, 123.58, 122.74, 117.60, 116.35, 74.39, 36.92, 22.29, 19.82. HRMS (ESI\(^+\)): \( m/z \) calcd for C\(_{31}\)H\(_{27}\)N\(_3\)O\(_6\)S, [M+H]\(^+\), 570.16; found 570.1694, C\(_{31}\)H\(_{27}\)N\(_3\)O\(_6\)SH [M+H]\(^+\).

1-(2-allylphenyl)allyl tetradecanoate (3g)
Prepared according to general procedure for esters (S2.2). Purified by silica gel column chromatography (9:1 cyclohexane: ethyl acetate). Pale yellow oil (85% yield \(3g\)) \(^1\)H NMR (500 MHz, Chloroform-\(d\)) \(\delta\) 7.43 – 7.36 (m, 1H), 7.26 – 7.23 (m, 2H), 7.22 – 7.15 (m, 1H), 6.50 (dd, \(J = 5.4, 1.6\) Hz, 1H), 5.98 (dddd, \(J = 16.6, 12.6, 10.3, 5.9\) Hz, 2H), 5.23 (dt, \(J = 5.1, 1.4\) Hz, 1H), 5.20 (dt, \(J = 11.5, 1.4\) Hz, 1H), 5.08 (dq, \(J = 10.1, 1.6\) Hz, 1H), 5.01 (dq, \(J = 17.1, 1.8\) Hz, 1H), 3.59 – 3.42 (m, 2H), 2.34 (td, \(J = 7.5, 3.3\) Hz, 2H), 1.62 (p, \(J = 7.3\) Hz, 2H), 1.34 – 1.20 (m, 21H), 0.88 (t, \(J = 6.9\) Hz, 3H). \(^{13}\)C NMR (126 MHz, Chloroform-\(d\)) \(\delta\) 172.74, 137.63, 137.05, 136.91, 136.28, 129.92, 128.21, 127.60, 126.65, 116.62, 116.14, 72.54, 36.86, 34.52, 31.93, 29.68, 29.65, 29.59, 29.46, 29.37, 29.26, 29.12, 24.94, 22.71, 14.13. HRMS (ESI\(^+\)): \(m/z\) calcd for \(C_{26}H_{40}O_2\), [M+H]\(^+\), 385.30; found 385.29, \(C_{26}H_{40}O_2\), [M+H]\(^+\), 407.29, \(C_{26}H_{40}O_2Na\) [M+H+Na]\(^+\).

S3.2.- Characterization of ethers 4a-b

7-((1-(2-allylphenyl)allyl)oxy)-2H-chromen-2-one (4a)

Prepared according to general procedure for ethers (S2.3). Purified by silica gel column chromatography (95:5 cyclohexane: ethyl acetate). Pale yellow oil (35% yield \(4a\)) \(^1\)H NMR (500 MHz, Chloroform-\(d\)) \(\delta\) 7.59 (d, \(J = 9.4\) Hz, 1H), 7.44 (dd, \(J = 7.7, 1.7\) Hz, 1H), 7.32 (d, \(J = 8.6\) Hz, 1H), 7.30 – 7.21 (m, 4H), 6.87 (dd, \(J = 8.6, 2.4\) Hz, 1H), 6.79 (d, \(J = 2.4\) Hz, 1H), 6.22 (d, \(J = 9.5\) Hz, 1H), 6.11 (dd, \(J = 17.2, 10.5, 5.5\) Hz, 1H), 6.04 – 5.95 (m, 1H), 5.93 (dt, \(J = 5.6, 1.5\) Hz, 1H), 5.35 – 5.28 (m, 2H), 5.15 (dq, \(J = 10.1, 1.5\) Hz, 1H), 5.06 (dq, \(J = 17.1, 1.7\) Hz, 1H), 3.55 – 3.44 (m, 2H). \(^{13}\)C NMR (126 MHz, Chloroform-\(d\)) \(\delta\) 161.20, 161.02, 155.66, 143.33, 137.06, 136.56, 136.44, 135.95, 130.34, 128.63, 128.46, 127.05, 126.93, 117.72, 116.73, 114.00, 113.19, 112.69, 103.04, 77.89, 37.49, 36.88. HRMS (ESI\(^+\)): \(m/z\) calcd for \(C_{21}H_{16}O_3\), [M+H]\(^+\), 319.13; found 319.11, \(C_{21}H_{16}O_3\), [M+H]\(^+\), 341.11, \(C_{21}H_{16}O_3Na\) [M+H+Na]\(^+\).

1-((1-(2-allylphenyl)allyl)oxy)-2,3,4,5,6-pentafluorobenzene (4b)
Prepared according to general procedure for ethers (S2.3). Purified by silica gel column chromatography (95:5 cyclohexane: ethyl acetate). Pale yellow oil (28% yield 4b) \( ^1H \) NMR (500 MHz, Chloroform-\( d \)) \( \delta \) 7.64 (dd, \( J = 7.3, 1.9 \) Hz, 1H), 7.31 (pd, \( J = 7.4, 1.7 \) Hz, 2H), 7.19 (dd, \( J = 7.2, 1.9 \) Hz, 1H), 6.08 (ddddt, \( J = 18.5, 9.8, 7.5, 1.1 \) Hz, 1H), 6.00 – 5.86 (m, 2H), 5.25 (dddd, \( J = 13.6, 3.7, 1.2 \) Hz, 2H), 5.06 (dq, \( J = 10.1, 1.6 \) Hz, 1H), 4.95 (dq, \( J = 17.1, 1.7 \) Hz, 1H), 3.46 (dt, \( J = 6.2, 1.7 \) Hz, 2H). \(^{13}C\) NMR (126 MHz, Chloroform-\( d \)) \( \delta \) 137.22, 136.63, 136.58, 135.93, 130.15, 128.73, 127.21, 127.00, 119.54, 116.24, 83.77, 36.72. HRMS (ESI\(^+\)): \( m/z \) calcd for \( \text{C}_{21}\text{H}_{18}\text{O}_3 \), [M+H]\(^+\), 319.13; found 319.11, \( \text{C}_{21}\text{H}_{18}\text{O}_3\text{Na} \), [M+H+Na]\(^+\).

S3.3.- Characterization of carbamates 5a-d

methyl 2-(((1-(2-allylphenyl)allyl)oxy)carbonyl)amino)benzoate (5a)

Prepared according to general procedure for carbamates (S2.4). Purified by silica gel column chromatography (9:1 cyclohexane: ethyl acetate). Off-white oil (68% yield 5a) \(^1H\) NMR (500 MHz, Chloroform-\( d \)) \( \delta \) 10.60 (s, 1H), 8.43 (dd, \( J = 8.6, 1.1 \) Hz, 1H), 8.00 (dd, \( J = 8.0, 1.7 \) Hz, 1H), 7.53 – 7.46 (m, 2H), 7.28 – 7.25 (m, 3H), 7.22 – 7.18 (m, 1H), 7.02 (ddd, \( J = 8.2, 7.3, 1.2 \) Hz, 1H), 6.51 (dt, \( J = 5.3, 1.6 \) Hz, 1H), 6.11 – 5.97 (m, 2H), 5.32 – 5.23 (m, 3H), 5.12 – 5.01 (m, 2H), 3.92 (s, 3H), 3.57 (dddd, \( J = 55.2, 16.0, 6.1, 1.6 \) Hz, 2H). \(^{13}C\) NMR (126 MHz, Chloroform-\( d \)) \( \delta \) 168.52, 152.67, 141.71, 137.60, 136.98, 136.95, 136.29, 134.57, 130.84, 129.92, 128.71, 127.71, 126.73, 121.58, 118.91, 116.81, 116.18, 114.55, 73.64, 52.27, 36.92. (ESI\(^+\)): \( m/z \) calcd for \( \text{C}_{21}\text{H}_{21}\text{N}_{2}\text{O}_4 \), [M+H]\(^+\), 352.15; found 352.14, \( \text{C}_{21}\text{H}_{21}\text{N}_{2}\text{O}_4\text{Na} \), [M+H+Na]\(^+\).

1-(2-allylphenyl)allyl (4-ethylphenyl)carbamate (5b)

Prepared according to general procedure for carbamates (S2.4). Purified by silica gel column chromatography (9:1 cyclohexane:ethyl acetate). Light yellow palettes (80% yield 5b) \(^1H\) NMR (500 MHz, Chloroform-\( d \)) \( \delta \) 7.46 – 7.38 (m, 1H), 7.30 – 7.26 (m, 4H), 7.23 – 7.19 (m, 1H), 7.14 – 7.09 (m, 2H), 6.60 (s, 1H), 6.51 (dt, \( J = 5.3, 1.6 \) Hz, 1H), 6.11 – 5.94 (m, 2H), 5.32 – 5.23 (m, 2H), 5.12 – 4.97 (m, 2H), 3.67 – 3.44 (m, 2H), 2.60 (q, \( J = 7.6 \) Hz, 2H), 1.20 (t, \( J = 7.6 \) Hz, 3H). \(^{13}C\) NMR (126 MHz, Chloroform-\( d \)) \( \delta \) 137.79, 136.99, 136.93, 136.28, 135.32, 130.07, 128.38, 128.37, 127.67, 126.68, 118.80, 116.18, 36.93, 28.21, 26.93, 15.70. (ESI\(^+\)): \( m/z \) calcd for \( \text{C}_{21}\text{H}_{21}\text{NO}_4 \), [M+H]\(^+\), 322.17; found 322.16, 344.16, \( \text{C}_{21}\text{H}_{23}\text{NO}_3 \), [M+H]\(^+\), \( \text{C}_{21}\text{H}_{23}\text{NO}_3\text{Na} \), [M+H+Na]\(^+\).

1-(2-allylphenyl)allyl (4-methyl-2-oxo-2H-chromen-7-yl)carbamate (5c)
Prepared according to general procedure for carbamates (S2.4). Purified by silica gel column chromatography (7:3 → 2:1 cyclohexane: ethyl acetate). White powder (33% yield 5c) $^1$H NMR (500 MHz, Acetone-$d_6$) δ 9.28 (s, 1H), 7.66 (d, $J = 8.7$ Hz, 1H), 7.61 (d, $J = 2.1$ Hz, 1H), 7.47 (dd, $J = 8.7$, 2.1 Hz, 1H), 7.41 (dd, $J = 7.1$, 2.1 Hz, 1H), 7.28 – 7.24 (m, 2H), 7.23 – 7.21 (m, 1H), 6.53 (dt, $J = 5.5$, 1.6 Hz, 1H), 6.16 – 5.94 (m, 3H), 5.33 – 5.21 (m, 2H), 5.08 – 4.97 (m, 2H), 3.65 – 3.47 (m, 2H), 2.41 (d, $J = 1.2$ Hz, 3H).

$^{13}$C NMR (126 MHz, Acetone-$d_6$) δ 159.83, 154.56, 152.53, 137.76, 137.23, 137.15, 136.54, 129.91, 128.29, 127.47, 126.62, 125.71 (d, $J = 1.2$ Hz), 116.11, 115.44, 114.95, 114.14 (d, $J = 9.1$ Hz), 112.44, 104.94 (d, $J = 8.6$ Hz), 73.33, 36.58, 26.62, 17.50. (ESI+): $m/z$ calcd for C$_{23}$H$_{21}$NO$_4$, [M+H]$^+$, 376.15; found 376.13, C$_{23}$H$_{21}$NO$_4$Na [M+H+Na]$^+$. 

1-(2-allylphenyl)allyl (4-chloro-3-(trifluoromethyl)phenyl)carbamate (5d)

Prepared according to general procedure for carbamates (S2.4). Purified by silica gel column chromatography (9:1 cyclohexane: ethyl acetate). Light yellow oil (78% yield 5d) $^1$H NMR (500 MHz, Chloroform-$d_6$) δ 7.72 (d, $J = 2.6$ Hz, 1H), 7.57 – 7.49 (m, 1H), 7.43 – 7.38 (m, 2H), 7.31 – 7.26 (m, 2H), 7.22 (dd, $J = 7.2$, 2.0 Hz, 1H), 6.79 (s, 1H), 6.51 (dt, $J = 5.4$, 1.5 Hz, 1H), 6.12 – 5.93 (m, 2H), 5.33 – 5.23 (m, 2H), 5.18 – 4.97 (m, 2H), 3.55 (dddt, $J = 54.8$, 16.1, 6.0, 1.7 Hz, 2H). $^{13}$C NMR (126 MHz, Chloroform-$d_6$) δ 164.21, 153.53, 151.02, 137.67, 137.14, 136.75, 136.52, 135.75, 130.14, 128.56, 127.64, 126.86, 126.15, 123.29, 117.32, 116.34, 73.93. 36.92. (ESI+): $m/z$ calcd for C$_{20}$H$_{17}$ClF$_3$NO$_2$, [M+H]$^+$, 396.09; found 396.09, C$_{20}$H$_{17}$ClF$_3$NO$_2$Na [M+H+Na]$^+$. 418.09, C$_{20}$H$_{17}$ClF$_3$NO$_2$Na [M+H+Na]$^+$. 

S4.-$^1$H NMR STUDIES OF THE RU-TRIGGERED UNCAGING REACTION OF ESTERS

S4.1.-Stability of the ester 3c

Substrate 3c was selected as model substrate to evaluate its stability in the reaction buffer and monitor the uncaging reaction. A solution of ester 3c (1 mg, 0.0025 mmol) was dissolved in 200 µL PBS buffer:acetone 4:1 containing 50 mM MgCl$_2$ at pH 6.0.
The mixture was incubated at 37 °C for 24 hours. After 24 hours, the mixture was concentrated under vacuum and analyzed by \(^1\)H NMR.

Before incubation (Figure S1): \(^1\)H NMR (500 MHz, Chloroform-\(d\)) \(\delta\) 8.40 (d, \(J = 1.7 \) Hz, 1H), 8.13 (dd, \(J = 8.4, 1.7 \) Hz, 1H), 7.84 (d, \(J = 8.4 \) Hz, 1H), 7.50 – 7.46 (m, 1H), 7.33 – 7.27 (m, 2H), 7.25 – 7.21 (m, 1H), 6.74 (dt, \(J = 5.6, 1.5 \) Hz, 1H), 6.13 (ddd, \(J = 17.1, 10.5, 5.5 \) Hz, 1H), 5.99 (ddt, \(J = 16.6, 10.2, 6.2 \) Hz, 1H), 5.35 – 5.27 (m, 2H), 5.08 (dq, \(J = 10.1, 1.6 \) Hz, 1H), 5.01 (dq, \(J = 17.1, 1.7 \) Hz, 1H), 3.65 – 3.47 (m, 2H).

After incubation (Figure S2): \(^1\)H NMR (500 MHz, Acetone-\(d_6\)) \(\delta\) 8.32 (d, \(J = 1.7 \) Hz, 1H), 8.16 (dd, \(J = 8.4, 1.8 \) Hz, 1H), 7.97 (d, \(J = 8.4 \) Hz, 1H), 7.46 (dd, \(J = 6.7, 2.5 \) Hz, 1H), 7.22 – 7.08 (m, 3H), 6.65 (dt, \(J = 5.8, 1.5 \) Hz, 1H), 6.10 (ddd, \(J = 17.3, 10.5, 5.6 \) Hz, 1H), 5.90 (ddt, \(J = 16.7, 10.2, 6.3 \) Hz, 1H), 5.30 – 5.15 (m, 2H), 4.98 – 4.85 (m, 2H), 3.52 (dd, \(J = 8.8, 7.1 \) Hz, 1H), 3.47 – 3.41 (m, 1H).

Figure S1. \(^1\)H NMR analysis of ester 3c before incubation at 37 °C.
Figure S2. $^1$H NMR analysis of ester 3c after 24-hour incubation at 37 °C
S4.2.- NMR studies of the uncaging reaction of ester 3c

A solution of ester 3c (1.0 mg, 0.0025 mmol) in PBS buffer:acetone 4:1 (200 µL, pH 6.0) was added to a 1.5 mL glass vial. To the mixture, was added \textbf{HG-II} (1 µL of a 10 mM stock solution in acetone) and the resulting mixture placed in a thermomixer (37 °C, 700 rpm for 24 h). The reaction mixture was concentrated under vacuum and analyzed by \textsuperscript{1}H NMR. The NMR spectra (Figure 3) highlights the formation of naphthalene 2 and the corresponding 3-bromo-4-nitrobenzoic acid 3c\_p in equimolar amounts. \textsuperscript{1}H NMR (500 MHz, Acetone-\textit{d}_6) δ 8.36 (d, \(J = 1.7\) Hz, 1H), 8.20 (dd, \(J = 8.4, 1.7\) Hz, 1H), 8.06 (d, \(J = 8.3\) Hz, 1H), 7.89 – 7.84 (m, 4H), 7.47 (dd, \(J = 6.3, 3.2\) Hz, 4H). Multiplicity of the 3-bromo-4-nitrobenzoic acid 3c\_p in the reaction mixture correspond to the multiplicity of the pure product (Figure 4-5).

\textbf{Figure S3. \textsuperscript{1}H NMR analysis of 3c after 24 hours reaction at 37 °C}
**Figure S4.** $^1$H NMR analysis of the product 3-bromo-4-nitrobenzoic acid 3c_p.

$^1$H NMR (500 MHz, Methanol-d$_4$) 8 8.41 (d, $J = 1.7$ Hz, 1H), 8.17 (dd, $J = 8.4, 1.7$ Hz, 1H), 7.97 (d, $J = 8.4$ Hz, 1H).

**Figure S5.** $^1$H NMR spectra of pure 3-bromo-4-nitrobenzoic acid 3c_p (top) and reaction mixture (bottom).

$^1$H NMR (500 MHz, Methanol-d$_4$) 8 8.36 (d, $J = 8.3$ Hz, 2H), 8.10 (dd, $J = 8.3, 1.8$ Hz, 2H), 7.96 (d, $J = 8.6$ Hz, 2H), 7.47 (s, 1H).
S5.-QUANTITATIVE GC-MS ANALYSIS

S5.1.-General procedure

In a glass vial (1.5 mL), catalyst **HG-II** (10 µL of a 100 µM stock solution in acetone) was added, followed by the addition of PBS buffer (90 µL). To the mixture is added the substrate (100 µL of a 2 mM stock solution in PBS buffer containing 10% acetone). The resulting mixture was stirred in a thermomixer at 37 °C. The reaction mixture was extracted with EtOAc (1 mL) containing 50 µM internal standard, and the organic phase was analyzed by GC-MS. Quantitative analysis of the uncaging efficiency was highlighted by the formation of the ring-closed product, i.e. naphthalene 2 (GC-MS settings are reported in S1).

S5.2.-Calibration curve of naphthalene 2 for GC-MS quantitative analysis

Calibration samples were prepared in triplicate containing naphthalene 2 with a concentration range spanning from 0-100 µM. Each calibration sample contained a fixed concentration (50 µM) of internal standard, 1-methoxynaphthalene. The linear regression obtained from the ratio between product concentration (X axis) and the ratio product/standard integration (Y axis) is used for determining the concentration of product in the corresponding reaction mixtures.

**Table S1. Calibration curve of naphthalene 2 using 1-methoxynaphthalene as internal standard**

| Entry | Product (µM) | Product/Standard |
|-------|--------------|------------------|
| 1     | 0            | 0                |
| 2     | 10           | 0.74574539       |
| 3     | 20           | 1.34811757       |
| 4     | 50           | 3.09954564       |
| 5     | 100          | 5.99228656       |
Figure S6. Linear regression for the calibration of naphthalene 2 using 1-methoxynaphtalene.

\[ y = 0.0607x \]
\[ R^2 = 0.9971 \]

S5.3.- Catalysts screening with 1-(2-allylphenyl)prop-2-en-1-ol (1)

Table S2. Screening of metathesis catalysts for the RCM-triggered elimination of water from 1

| Entry | Catalyst  | Product integration (AVG) | Standard (1-methoxynaphthalene) integration (AVG) | Conversion% | TON |
|-------|-----------|---------------------------|--------------------------------------------------|-------------|-----|
| 1     | G-II      | 82450                     | 113974                                           | 6±0         | 12±1|
| 2     | Aquamet   | 310113                    | 122262                                           | 21±0        | 42±1|
| 3     | HG-II     | 279943                    | 117698                                           | 20±2        | 39±5|
| 4     | HG-I      | 12191                     | 121504                                           | 1±0         | 2±0 |

*Results are the average of three independent experiments. [1] = 1.0 mM. PBS buffer includes 50 mM MgCl₂.*

Table S3. Screening of metathesis catalysts for the RCM-triggered elimination of water from 1 at pH 7.4

1. G-II: 82450, 113974, 6±0, 12±1
2. Aquamet: 310113, 122262, 21±0, 42±1
3. HG-II: 279943, 117698, 20±2, 39±5
4. HG-I: 12191, 121504, 1±0, 2±0

These results indicate the catalytic efficiency of different metathesis catalysts in the elimination of water from 1 at pH 7.4.
S5.4.- Quantitative GC-MS analysis of esters 3, ethers 4, carbamates 5 and carbonates 6.

Table S4. Quantitative GC-MS analysis of naphthalene 2 resulting from the metathesis of substrates 3a-g, 4b, 5a-d, 6

| Entry | Substrate | mol%  | HG-II | Product integration (AVG) | Standard (1-methoxynaphthalene) integration (AVG) | Conversion % | TON |
|-------|-----------|-------|-------|---------------------------|--------------------------------------------------|--------------|-----|
| 1     | 3a        | 0.5   | 30895 | 46823                     | 5±1                                              | 11±3         |
| 2     | 3b        | 0.2   | 522848| 80674                     | 43±0                                             | 214±2        |
| 3     | 3c        | 0.2   | 617572| 77981                     | 52±1                                             | 261±7        |
| 4     | 3d        | 0.1   | 311409| 78841                     | 26±2                                             | 260±20       |
| 5     | 3e        | 0.2   | 172468| 74045                     | 15±0                                             | 77±0         |
| 6b    | 3f        | 10.0  | 90337 | 871456                    | 43±7                                             | 4±1          |
| 7     | 3g        | 0.2   | 493655| 69006                     | 47±1                                             | 235±5        |
| 8     | 4b        | 0.5   | 311705| 57920                     | 45±5                                             | 90±10        |
| 9     | 5a        | 0.2   | 119163| 99425                     | 10±0                                             | 49±0         |
| 10    | 5b        | 0.2   | 241074| 94109                     | 17±1                                             | 84±5         |
| 11c   | 5c        | 1.0   | 25413 | 89085                     | 9±0                                              | 9±0          |
| 12    | 5d        | 0.05  | 32417 | 109875                    | 5±1                                              | 97±11        |
| 13    | 6         | 0.2   | 105419| 79377                     | 9±1                                              | 44±2         |

*Results are the average of two independent experiments. [1] = 1.0 mM. PBS buffer includes 50 mM MgCl₂.

| Entry | Substrate | Product integration (Average) | Standard (1-methoxynaphthalene) integration (AVG) | Conversion% | TON |
|-------|-----------|-------------------------------|--------------------------------------------------|--------------|-----|
| 1     | 3b        | 138222                        | 58331                                            | 20±5         | 20±5 |
| 2     | 3c        | 94068                         | 58292                                            | 13±1         | 13±1 |
| 3     | 3d        | 44417                         | 53076                                            | 7±2          | 7±2  |
| 4     | 3e        | 193444                        | 43467                                            | 37±6         | 37±6 |
| 5     | 3g        | 177417                        | 54132                                            | 27±0         | 27±0 |
| 6     | 4b        | 37205                         | 56670                                            | 5±1          | 5±1  |
| 7     | 5b        | 93655                         | 47437                                            | 16±0         | 16±0 |

*Results are the average of two independent experiments. Substrate concentration: 1.0 mM. PBS buffer does not include 50 mM MgCl₂.
S5.5.- Monitoring the uncaging reaction of ester 3d over time

Table S6. RCM-triggered elimination of 3d. Data points at 5, 10, 30 and 60 min

| Entry | Time (min) | Product integration (AVG) | Standard (1-methoxynaphthalene) integration (AVG) | Conv.% | TON |
|-------|------------|---------------------------|-----------------------------------------------|--------|-----|
| 1     | 0          | —                         | —                                             | —      | —   |
| 2     | 5          | 513777                    | 80580                                         | 42±2   | 105±4 |
| 3     | 10         | 541806                    | 81934                                         | 44±0   | 109±0 |
| 4     | 30         | 602122                    | 78870                                         | 50±2   | 126±4 |
| 5     | 60         | 1349515                   | 82853                                         | 54±3   | 135±7 |

*[^3d] = 2.5 mM, [HG-II] = 50 µM. Reaction media: PBS buffer:acetone 3:1, 50 mM MgCl₂, pH 6

Figure S7. Time-dependent quantitative analysis of product 2 from the RCM of ester 3da

S6.- FLUORESCENCE EXPERIMENTS OF FLUOROGENIC SUBSTRATES 4a AND 5c.

S6.1.- RCM-triggered uncaging of fluorogenic ether 4a

In a 1.5 mL glass vial, ether 4a (5 µL of a 20 mM stock solution in DMSO) was added to a solution of PBS buffer (190 µL). Catalyst HG-II (5 µL of a 100 µM stock solution in DMSO) was added to the mixture and let to shake in a thermomixer at 37 °C for 48 h. An aliquot of the reaction mixture (12.5 µL) was collected and transferred to a 96-well plate (Nunclon 96 flat black) containing PBS buffer (187.5 µL, 16-fold dilution).
The plate was analyzed by fluorescence (excitation: 325nm, emission: 450 nm, bandwidth interval 5.0 nm, settle time 0.1 s.

Table S7. Calibration curve of umbelliferone 7

| Entry | umbelliferone 7 (µM) | Fluorescence intensity (a.u.) |
|-------|----------------------|------------------------------|
| 1     | 20                   | 18128                        |
| 2     | 15                   | 14249                        |
| 3     | 10                   | 9782                         |
| 4     | 5                    | 5184                         |
| 5     | 0                    | 0                            |

Figure S8. Linear regression for the calibration of umbelliferone 7 in PBS buffer

Table S8. Fluorescence assay of the RCM-triggered elimination of ether 4a

| Entry | Substrate (mM) | Catalyst (mol%) | Time (h) | Fluorescence Intensity | Conv% | TON  |
|-------|----------------|-----------------|----------|------------------------|-------|------|
| 1     | 0.5            | —               | 48       | 793                    | 0.3   | 0    |
| 2     | 0.5            | Aquamet (0.5)   | 48       | 4039                   | 7.0   | 14   |
| 3     | 0.5            | HG-II (0.5)     | 48       | 9281                   | 32.0  | 64   |

*Reaction conditions reported above (S6.1).
S6.2.- Kinetic studies of the uncaging reaction of substrate 4a

Table S9. Kinetic data of the RCM-triggered elimination of substrate 4a

| Time (min) | HG-II     | Aquamet (AM) | Blankb |
|-----------|-----------|--------------|--------|
| 1         | 508.5± 71.5 | 684.5±64.5   | 351    |
| 5         | 2630±190   | 935±94       | 338    |
| 10        | 6870.5±298.5 | 1097±115    | 339    |
| 15        | 11065.5±293.5 | 1274.5±138.5 | 335    |
| 20        | 14307±343  | 1440.5±163.5 | 332    |
| 25        | 16851±489  | 1619±187     | 335    |
| 30        | 19108±990  | 1823.5±211.5 | 330    |
| 35        | 21082.5±165.5 | 2061±234   | 346    |
| 40        | 21885±127  | 2342±246     | 348    |
| 45        | 22621±175  | 2634±262     | 349    |
| 50        | 23020.5±113.5 | 2978.5±284.5 | 353    |
| 55        | 23314.5±94.5 | 3520.5±255.5 | 355    |
| 60        | 23780.5±128.5 | 3891.5±215.5 | 359    |
| 65        | 23881±75   | 4237.5±246.5 | 360    |
| 70        | 24081.5±60.5 | 4501.5±233.5 | 361    |
| 75        | 24185±76   | 4721±236     | 365    |
| 80        | 24449.5±21.5 | 4911±228    | 371    |
| 85        | 24487.5±217.5 | 5057±243   | 371    |
| 90        | 24601±110  | 5225±242     | 373    |
| 95        | 24579.5±165.5 | 5350.5±252.5 | 376    |
| 100       | 24781.5±173.5 | 5458.5±253.5 | 380    |
| 105       | 24804.5±219.5 | 5549±253    | 380    |
| 110       | 24821.5±212.5 | 5627±257    | 381    |
| 115       | 24782±208  | 5727.5±262.5 | 389    |
| 120       | 24926.5±196.5 | 5780±239    | 389    |

a [4a] = 100 µM, [HG-II] = [Aquamet] = 1 µM. Reaction media: PBS buffer, 50 mM MgCl2, pH 6, 1% DMSO. b Blank: 100 µM substrate 4a in the reaction media.
S6.3.- RCM-triggered uncaging of substrate 5c

In a 1.5 mL glass vial, carbamate 5c (4 µL of a 5 mM stock solution in DMSO) was added to a solution of PBS buffer (195 µL). Catalyst HG-II (1 µL of a 100 µM stock solution in DMSO) was added to the mixture and shaken in a thermomixer (37 °C, 1h). The reaction was transferred to a 96-well plate (Nunclon 96 flat black) and analyzed by fluorescence (excitation: 325nm, emission: 450 nm, bandwidth interval 5.0 nm, settle time 0.1 s). Fluorescence intensity (average of two independent experiments): 9380, 9±0% yield, 9±0 TON. Data consistent with the GC-MS quantitative analysis (see Table S4, entry 11).
Table S10. Calibration curve of 4-methyl-7-aminocoumarin 8

| Entry | 8 (µM) | Fluorescence Intensity (a.u.) |
|-------|--------|------------------------------|
| 1     | 100    | 9380                         |
| 2     | 10     | 1112                         |
| 3     | 1      | 134                          |
| 4     | 0.1    | 32                           |

Figure S10. Linear regression for the calibration curve of 4-methyl-7-aminocoumarin 8

\[ y = 93.976x \]
\[ R^2 = 0.9995 \]

Table S11. RCM-triggered release of 3d and 4a in complex biological media

| Entry | Substrate | mol% HG-II | Media | Conv% | TON  |
|-------|-----------|------------|-------|-------|------|
| 1     | \[ \text{ } \] | 0.2        | DMEM  | 14±1  | 69±2 |
| 2     | \[ \text{ } \] | 10.0       | DMEM  | 45±10 | 5±1  |
| 3     | \[ \text{ } \] | 0.5        | cell lysate | 5±0.5 | 10±1 |
S7.1.- General procedure

A solution of carboxylic acid 3d or ether 4a (1 µL of a 20 mM stock solution in DMSO) is added to the reaction media (198 µL) and mixed gently at room temperature, followed by the addition of HG-II (1 µL of a 100 µM stock solution in DMSO).

S7.2.- RCM of ether 4a in DMEM

The substrate solution was prepared as reported in the general procedure (S7.1) and added directly in a Nunclon 96 Flat Black. After the addition of catalyst, the plate was inserted in a Tecan reader (Infinite M1000Pro) and analyzed by fluorescence (Temperature 37 °C, kinetic cycle: 14, kinetic interval: 5.00 min, excitation 325nm, emission 450 nm, bandwidth interval 5.0 nm, settle time 0.1 s).

![Diagram of reaction](image)

Table S12. Calibration curve of umbelliferone 7 in DMEM

| Entry | 7 (µM) | Fluorescence Intensity (a.u.) |
|-------|--------|------------------------------|
| 1     | 40     | 18910±189                    |
| 2     | 20     | 10774±24                     |
| 3     | 10     | 5731±34                      |
| 4     | 5      | 3067±25                      |
| 5     | 0      | 100±2                        |
Figure S11. Linear regression for the calibration of umbelliferone 7 in DMEM

![Linear regression graph](image.png)

\[ y = 491.55x \]
\[ R^2 = 0.9885 \]

Figure S12. Fluorescence analysis of the RCM-triggered 1,4-elimination of 4b in DMEM

![Fluorescence analysis graph](image.png)

a No catalyst legend: 100 µM ether 4a in the reaction media. Blank legend: only reaction media

S7.3. RCM of ether 4a in *E. coli* cell lysates

![Chemical reaction](image.png)

Table S13. Calibration curve of umbelliferone 7 in cell lysate

| Entry | 7 (µM) | Fluorescence Intensity (a.u.) |
|-------|-------|-------------------------------|
| 1     | 30    | 26100±2673                    |
Figure S13. Linear regression for the calibration of umbelliferone 7 in cell lysate

\[
y = 899.05x \\
R^2 = 0.9931
\]

Figure S14. Results of the fluorescence assay for the RCM-triggered elimination of 4a in cell lysate

S7.4.-RCM of ether 4a in the presence of E. coli cells
*E. coli* cells (Top10) were cultured in LB medium supplemented with Kanamycin (50 µg/mL) and incubated at 37 °C for 16 hours. After incubation, cells with an O.D._600_ = 3 of cells were transferred to Eppendorf tubes and centrifuged (14’500 g, 10 min, 4 °C), the supernatant was discarded and the cell pellet was washed with cold PBS buffer, centrifuged and resuspended in the reaction media (198 µL PBS buffer, 50 mM MgCl$_2$, pH 6) and transferred to a 1.5 mL glass vial or to a 96-well plate. The substrate 4b (1 µL, 20 mM solution in DMSO) is added to the mixture, followed by the addition of HG-II (1 µL of a 200 µM solution in DMSO). The resulting mixture is stirred for 1 hour at 37 °C or is followed over time at a plate reader.

**Table S14. Fluorescence intensity values for the calibration curve of umbelliferone in cellular media**

| Umbelliferon (µM) | calibration 1 | calibration 2 | calibration AVG |
|------------------|--------------|--------------|----------------|
| 100              | 39344        | 39773        | 39558.5        |
| 50               | 19485        | 19690        | 19587.5        |
| 30               | 11515        | 11742        | 11628.5        |
| 20               | 7782         | 7957         | 7869.5         |
| 10               | 4092         | 3906         | 3999           |
| 5                | 2163         | 2112         | 2137.5         |
| 0                | 140          | 144          | 142            |

**Figure S15. Calibration curve of umbelliferone in cellular media**

\[ y = 394.41x \]
\[ R^2 = 0.9999 \]
Table S15. Results of the RCM-triggered uncaging of 4a in the presence of cells

| Time (s) | Product (µM) = TON ± error | blank\textsuperscript{a} (µM) |
|----------|----------------------------|-----------------------------|
| 0        | 0.375244 ±0.00254          | 0.373976                    |
| 300      | 1.157425 ±0.051976         | 0.343551                    |
| 600      | 4.149236 ±0.084937         | 0.342283                    |
| 900      | 9.895794 ±0.167339         | 0.339748                    |
| 1200     | 16.63244 ±0.076063         | 0.341016                    |
| 1500     | 22.17489 ±0.152126         | 0.399331                    |
| 1800     | 25.85 ±0.0824              | 0.352425                    |
| 2100     | 28.39558 ±0.128039         | 0.380315                    |
| 2400     | 29.86866 ±0.1255           | 0.34989                      |
| 2700     | 30.77635 ±0.1255           | 0.413276                    |
| 3000     | 31.51543 ±0.00254          | 0.368905                    |
| 3300     | 31.97561 ±0.036764         | 0.395527                    |
| 3600     | 32.5296 ±0.04057           | 0.377779                    |
| 3900     | 32.7692 ±0.06719           | 0.38285                      |

\textsuperscript{a}Blank refers to the reaction carried out only in the presence of 4a (100 µM).
Figure S16. RCM-triggered uncaging of substrate 4a in the presence of *E. coli* cells

Figure S17. Close-to-release reaction in the periplasm of *E.coli*
General procedure (adapted from the literature)²

*E. coli* cells (Top10) were cultured in LB-medium supplemented with kanamycin (50 µg/mL) at 37 °C for 16 hours. ZYM 5052 medium (25 mL) supplemented with kanamycin (50 µg/mL) in baffled flasks were inoculated with an OD$_{600}$ = 1 and incubated at 37 °C. After 3.5 hours, IPTG (0.25 mL, 50 mM) was added to the cell cultures and incubated at 18 °C for 18 hours. After Sav expression, the cells were transferred to Eppendorf tubes (normalized to O.D.$_{600}$ = 3) and centrifuged (14’500 g, 10 min, 4 °C), the supernatant was discarded and the cell pellet washed with cold PBS buffer, centrifuged and resuspended in PBS buffer containing **Biot-Ru** (500 µL PBS buffer, 10 µM **Biot-Ru**) and incubated on ice for 30 min. After incubation, the cells were centrifuged, washed with cold PBS buffer, re-centrifuged, resuspended in the reaction media containing **4a** (200 µL of a 100 µM solution in PBS buffer, 50 mM MgCl$_2$, pH 6) and transferred to 1.5 mL glass vials. The glass vials were placed in a thermomixer at 37 °C, 800 rpm. After 1 hour, 100 µL of the reaction mixture was transferred to a 96-well plate containing 100 µL reaction media and analyzed by fluorescence.
Table S16. Calibration curve for the quantification of umbelliferone by fluorescence in cellular media ($\lambda_{\text{excitation}}$ 325 nm, $\lambda_{\text{emission}}$ 450 nm)

| Product (µM) | fluorescence intensity (AVG) | error |
|--------------|-------------------------------|-------|
| 20           | 12830 ± 11.5                  |       |
| 10           | 6148 ± 32                     |       |
| 5            | 3820 ± 12.5                   |       |
| 0            | 112 ± 3.5                     |       |

Figure S18. Calibration curve for the quantification of umbelliferone by fluorescence in cellular media ($\lambda_{\text{excitation}}$ 325 nm, $\lambda_{\text{emission}}$ 450 nm)
Figure S19. RCM-triggered uncaging of substrate 4a in the periplasm of *E. coli* expressing WT Sav<sub>peri</sub>

Table S17. Fluorescence intensity values resulting from the uncaging reaction of substrate 4a

| Entry    | Fluorescence Intensity (a.u.) | Product (µM) | Product (µM) 2-fold dilution |
|----------|-------------------------------|--------------|-------------------------------|
| Blank*   | 241.5                         | 0.38         | 0.8±0.0                       |
| Empty**  | 936.5                         | 1.46         | 2.9±0.3                       |
| WT       | 8560                          | 13.33        | 26.7±5.6                      |

* Reaction media containing only substrate in the presence of *E. coli* cells. ** Empty is referred to the *E. coli* strain not expressing Sav. The results are the average of two independent experiments.

S9.- RCM-TRIGGERED UNCAGING OF UMBELLIFERONE IN THE PRESENCE OF MAMMALIAN CELLS

S9.1.- General procedure

HeLa cells were seeded in a 96-well plate (15,000 cells/well) and incubated at 37 °C for 24 hours in DMEM + 10 % FBS. After incubation, DMEM was removed from the wells and the cells were washed with PBS buffer, followed by the addition of 199 µL
of a solution of 4a in DMEM (or DMEM + 10% FBS). After 15 min incubation of the substrate at room temperature, the catalyst HG-II (1 µL, 200 µM in DMSO) is added to the mixture and the 96-well plate were incubated at 37 °C in a Tecan fluorimeter (Kinetic cycles: 16, kinetic interval: 10.00 min, λ_{excitation} 325 nm, λ_{emission} 450 nm).

**Figure S20. Calibration curve for the quantification of umbelliferone in DMEM FBS 10% in the presence of HeLa cells (λ_{excitation} 325 nm, λ_{emission} 450 nm)**

**Figure S21. Calibration curve for the quantification of umbelliferone in DMEM FBS 10% in the presence of HeLa cells (λ_{excitation} 325 nm, λ_{emission} 450 nm)**

**Table S18. Quantification of umbelliferone by fluorescence in DMEM with HeLa cells at pH 7.4 and pH 6.0 (λ_{excitation} 325 nm, λ_{emission} 450 nm)**

| Entry | F.I. (DMEM, pH 7.4) | F.I. (DMEM, pH 6.0) |
|-------|---------------------|---------------------|
|       | Blank | HG-II, 1 µM | HG-II, 5 µM | HG-II, 10 µM | blank | HG-II, 1 µM | HG-II, 5 µM | HG-II, 10 µM |
| 1     | 378   | 7460       | 19193      | 20990       | 334   | 8553       | 23116      | 22876       |
| 2     | 356   | 6878       | 19752      | 21228       | 304   | 9974       | 22892      | 23031       |
Table S19. Quantification of umbelliferone by fluorescence in DMEM + FBS 10% with HeLa cells at pH 7.4 and pH 6.0 ($\lambda_{\text{excitation}}$ 325 nm, $\lambda_{\text{emission}}$ 450 nm)

| Entry | F.I. (DMEM + FBS 10%, pH 7.4) | F.I. (DMEM + FBS 10%, pH 6.0) |
|-------|-------------------------------|-------------------------------|
|       | Blank  | HG-II, 1 µM | HG-II, 5 µM | HG-II, 10 µM | blank | HG-II, 1 µM | HG-II, 5 µM | HG-II, 10 µM |
| 1     | 880   | 2926       | 10574      | 13289       | 854   | 3142       | 12105       | 14913       |
| 2     | 453   | 3003       | 11283      | 13986       | 819   | 3361       | 12180       | 14149       |
| 3     | 775   | 3161       | 10598      | 13771       | —     | 3016       | 11763       | 13255       |
| AVG   | 703   | 3030       | 10818      | 13682       | 837   | 3173       | 12016       | 14106       |
Figure S22. RCM-triggered uncaging of umbelliferone in the presence of mammalian cells (end-point fluorescence measurements, \( \lambda_{\text{excitation}} 325 \text{ nm}, \lambda_{\text{emission}} 450 \text{ nm} \))

Figure S23. Kinetics of the RCM-triggered release of 4a in the presence of HeLa cells

S9.2.- Cell Viability Determination

HeLa cells were seeded in a 96-well plate (15’000 cells/well) and grown at 37 °C for 24h. Following 24h, the cells were treated with serial dilutions of substrate 4a, products
naphthalene and umbelliferone (0-100 µM) and **HG-II** (0-10 µM) in DMEM + 10% FBS. The final DMSO concentration in all samples was below 1% (v/v). After 24 h of incubation, the cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.³ The growth medium was removed and replenished with 100 µL of fresh DMEM + 10 % FBS supplemented with MTT (0.5mg/mL). Following incubation for 4h, the growth medium was removed and the resulting formazan dissolved by the addition of 100 µL DMSO. The plates were shaken at 500 RPM, 2mm throw for 10min at room temperature and the absorbance was determined at 575 nm. A 100% cell viability was determined from wells containing only DMEM + 10% FBS.

**Figure S24. MTT cell-viability assay of substrate 4a and products (umbelliferone and naphthalene) after 24 h incubation at 37 °C**
Figure S25. MTT viability assay of catalyst HG-II after 24 incubation at 37 °C

NMR AND HR-MS SPECTRA

S10.-
ester with nicotinic acid
esterif with nicotine acid

esterif with 2-chlorobenzonic acid
Esterif with 2-chlorobenzoic acid
esterification SAV 144 with 3-bromo-4-nitrobenzoic acid
Esterification with trifluoroacetylbenzoic acid, column product
Esterification with 4-trifluoracetylbenzoic acid column product
High Resolution Mass Spectrometry Report

Sample Name: 3a
Comment: 
Instrument: maXis 4G
Method: ms_nocolumn_300-600_pos.m

Acquisition Date: 14.06.2019 12:42:50
Page Bruker Compass DataAnalysis 4.0 1 of 3
Sample Name: 3b
Instrument: maXis 4G
Comment: 
Method: ms_nocolumn_300-600_pos.m

High Resolution Mass Spectrometry Report

Acquisition Date: 14.06.2019 12:45:55
Page Bruker Compass DataAnalysis 4.0 1 of 6
High Resolution Mass Spectrometry Report

Sample Name: 3c
Comment: 
Instrument: maXis 4G
Method: ms_nocolumn_300-600_pos.m

**Acquisition Date:** 14.06.2019 12:49:00

**Bruker Compass DataAnalysis 4.0**

---

**Page 1 of 3**
High Resolution Mass Spectrometry Report

Sample Name: 3d
Comment: 
Instrument: maXis 4G
Method: ms_nocolumn_300-600_pos.m

Acquisition Date: 14.06.2019 12:52:05
Page Bruker Compass DataAnalysis 4.0 1 of 3
High Resolution Mass Spectrometry Report

Sample Name: 3e
Comment: Instrument: maXis 4G
Method: ms_nocolumn_300-600_pos.m

**Acquisition Date:** 14.06.2019 12:55:10

Bruker Compass DataAnalysis 4.0
Page 1 of 3
High Resolution Mass Spectrometry Report

Sample Name  3f
Comment      Instrument  maXis 4G
Method       Sample Name  3f
Comment      Method  ms_nocolumn_300-600_pos.m

Acquisition Date  14.06.2019 12:58:15
Page Bruker Compass DataAnalysis 4.0  1 of 3
High Resolution Mass Spectrometry Report

Sample Name: 5b
Instrument: maXis 4G
Comment
Method: ms_nocolumn_300-600_pos.m

Acquisition Date: 14.06.2019 13:13:40
Page: 1 of 3
High Resolution Mass Spectrometry Report

Sample Name: 5d  
Comment:  
Instrument: maXis 4G  
Method: ms_nocolumn_300-600_pos.m

Acquisition Date: 14.06.2019 13:19:51  
Page Bruker Compass DataAnalysis 4.0 1 of 3

---

**Acquisition Data**

**Sample Name:** 5d  
**Comment:**  
**Instrument:** maXis 4G  
**Method:** ms_nocolumn_300-600_pos.m

**Acquisition Date:** 14.06.2019 13:19:51  
**Page:** Bruker Compass DataAnalysis 4.0 1 of 3
S11.- STABILITY OF ETHERS, CARBAMATES AND CARBONATES IN THE REACTION MEDIA

S11.1 - General procedure for the evaluation of the stability of model substrates 4b, 5c, and 6. a

Substrate 4b/5c/6 (1.0 mg dissolved in 0.6 mL solution of PBS buffer:acetone 3:1 containing 50 mM MgCl₂, pH 6). is stirred in a thermoshaker for a period of 1-24 hours at 37 °C. After 24 hours, the reaction mixture is concentrated under high vacuum and analyzed by ¹H NMR.

a The monitoring of the stability of the ester substrate 3c is presented in Figures S1 and S2.

S11.2 - ¹H NMR spectra of the substrates 4b, 5c and 6 after 1-hour and 24-hours

As can be appreciated from the spectra, the ether 4b and the carbonate 6 show no degradation after one hour (corresponding to the reaction time, Figure S26 and S27), but do show some degradation after 24-hour at 37 °C in PBS buffer at pH 6 (Figure S28 and S29). In stark contrast, carbamate 5c displays excellent stability even after 24 hours (Figure S30).
Figure S26. Stability of the ether 4b after 1-hour stirring at 37 °C, pH 6
Figure S27. Stability of the carbonate 6 after 1-hour stirring at 37 °C, pH 6

Figure S28. Stability of the carbonate 6 after 24-hour stirring at 37 °C, pH 6
Figure S29. Stability of the ether 4b after 24-hour stirring at 37 °C, pH 6

Figure S30. Stability of the carbamate 5c after 24-hour stirring at 37 °C, pH 6
S12.-REFERENCES

1. Yimiao, H.; Xuelian, W.; Jun-An, X.; Jinying, P.; Chunfang, G.; Yanmin, H.; Chuseng, H.; Metal-Free Oxidative Isocyanides Insertion with Aromatic Aldehydes to Aroylated N-Heterocycles. RSC Adv. 2018, 8, 3036-3040.

2. Jeschek, M.; Reuter, R.; Heinisch, T.; Trindler, C.; Klehr, J.; Panke, S.; Ward, T.R. Directed Evolution of Artificial Metalloenzymes for In Vivo Metathesis. Nature 2015, 537, 661-665.

3. (a) Riss Tl Fau - Moravec, R. A.; Moravec Ra Fau - Niles, A. L.; Niles Al Fau - Duellman, S.; Duellman S Fau - Benink, H. A.; Benink Ha Fau - Worzella, T. J.; Worzella Tj Fau - Minor, L.; Minor, L., Cell Viability Assays BTI - Assay Guidance Manual. (b) Berridge, M. V.; Herst, P. M.; Tan, A. S. Tetrazolium Dyes as Tools in Cell Biology: New Insights Into Their Cellular Reduction. In Biotechnol. Ann. Rev., Elsevier: 2005, 11, 127-152.