

Introducing a Squaramide-Based Self-Immolative Spacer for Controlled Drug Release

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Supporting Information

1. Materials and general methods

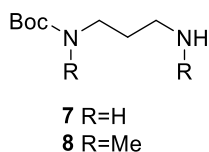
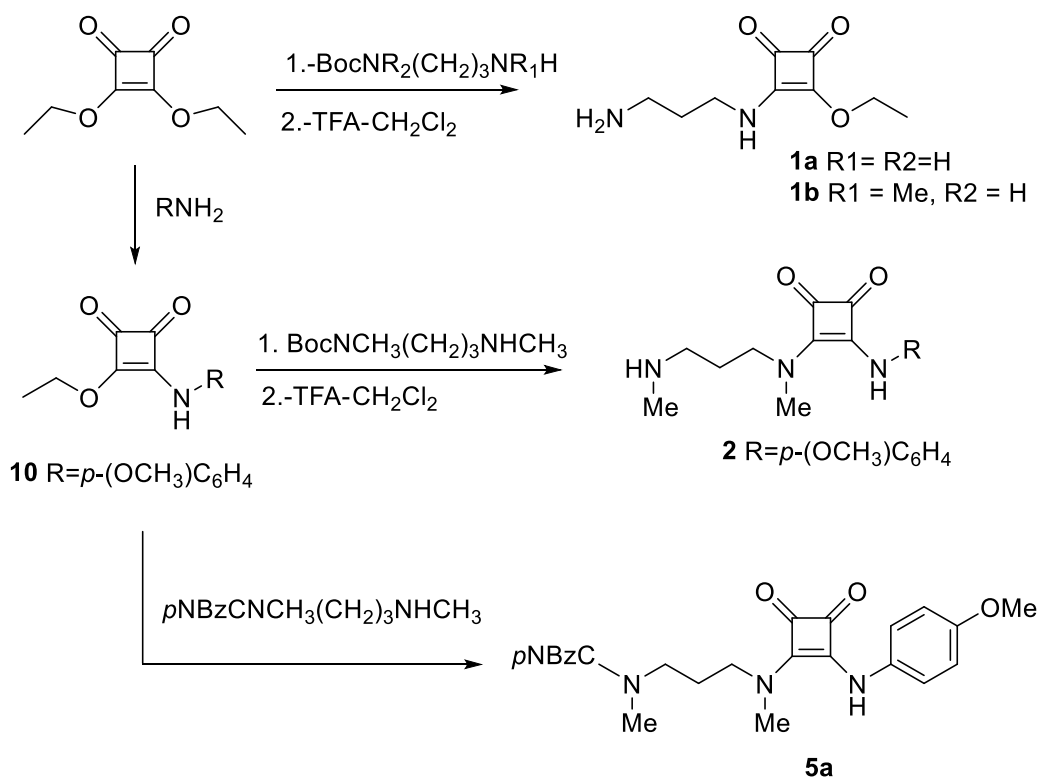
Chemicals were of commercial origin (Aldrich or Scharlau) and were used as received. ¹H, ¹³C and 2D NMR spectra (at 300 and 600 MHz) and ¹³C (at 75 and 150 MHz) spectra were recorded on 300 and 600 MHz spectrometers in CDCl₃, D₂O or DMSO-*d*₆ solutions at room temperature. The residual proton signal was used as a reference. Chemical shifts (δ) are given in ppm and coupling constants (*J*) in Hz. Splitting patterns are designated as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and br (broad). ESI-HRMS mass spectra were recorded on a magnetic sector on an Orbitrap mass spectrometer.

UV-vis experiments were performed on a VARIAN Cary 300 Bio UV-vis spectrophotometer at 37.0 ± 0.1 °C in water. Squaramate esters were dissolved in 10⁻² M buffered solutions (formic acid, acetic acid, sodium cacodylate, PBS and borate) at a final concentration of 30 μM. The ionic strength was 0.15 M NaCl. The changes in the UV range were analysed with the ReactLab™ Kinetics software (Jplus Consulting Ltd.).

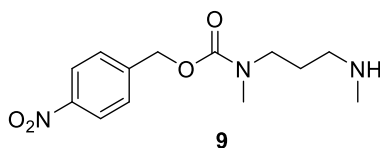
Kinetics and rate of release studies were performed with a Waters 600 HPLC pump, a PDA996 detector, a Millipore Waters 700 Satellite WISP autosampler, and a Waters XBridge C18 5 μm column (4.6 × 150 mm). The column was equilibrated in 95:5 H₂O (0.1 % Formic acid)/MeCN at 1 mL/min flow rate. After injection of the sample, a solvent gradient was established by ramping to 40:60 H₂O (0.1 % Formic acid)/MeCN over 20 minutes.

2. Experimental procedures and data for compounds

Scheme S1: Synthesis of ethyl squaramate **1**, squaramide **2** and **5a**

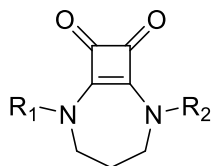


tert-butyl (3-aminopropyl)carbamate (7), and **tert-butyl methyl(3-(methylamino)propyl)carbamate (8)**. The products were prepared according to a reported procedure. Analytical data are in accordance with those reported on the literature.¹



4-nitrobenzyl methyl(3-(methylamino)propyl)carbamate (9). The product was prepared following a reported procedure.² Pale oil, 1230 mg (yield 89 %). ¹H NMR (300 MHz, CDCl₃) δ :

1.74 (m, 2.4H), 2.41 (s, 3.1H), 2.57 (t, $J = 7.0$ Hz, 2H), 2.96 (s, 2.9H), 3.38 (t, $J = 7.1$ Hz, 2.2H), 5.22 (s, 2H), 7.52 (d, $J = 8.9$ Hz, 1.7H), 8.22 (d, $J = 8.9$ Hz, 1.6H) ppm.

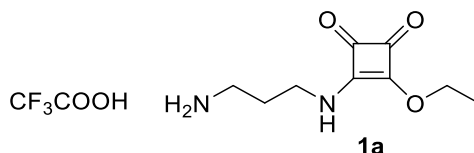


4a $R_1 = R_2 = H$

4b $R_1 = R_2 = Me$

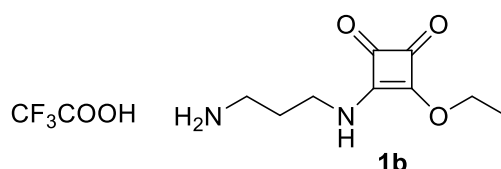
4c $R_1 = Me, R_2 = H$

2,6-diazabicyclo [5.2.0] non-1 (7) -ene-8,9-dione, (4a), 2,6-dimethyl-2,6-diazabicyclo [5.2.0]non-1(7)-ene-8,9-dione (4b), 2-Methyl-2,6-diazabicyclo[5.2.0]non-1(7)-ene-8,9-dione (4c). The products were prepared according to a reported procedure. Analytical data are in accordance with those reported on the literature.³



3-((2-ethoxy-3,4-dioxocyclobut-1-en-1-yl) amino) propan-1-aminium-trifluoroacetate (1a). The product was prepared according to a reported procedure.⁴ Diethyl squarate (500 mg, 2.94 mmol) in CH_3CN (3 mL) and one equivalent of the Boc-protected diamine were mixed in a round-bottom flask and the mixture was stirred under nitrogen at room temperature for 16 h. The crude mixture was concentrated to dryness under reduced pressure and purified by silica-gel column chromatography (dichloromethane/tetrahydrofuran gradient = 20:1) to afford the Boc-protected ethyl squaramate **1a** as a pale oil, 479 mg, 91% yield. 1H -NMR (300 MHz, $CDCl_3$): δ 1.44 (s, 9H), 1.46 (t, $J = 6.9$ Hz, 3H), 1.72 (m, 2H), 3.23 (br s, 2H), 3.47 (br s, 1.1H), 3.69 (br, 0.9H), 4.77 (q, $J = 6.9$ Hz, 2H), 6.68 (br, 1H), 6.93 (br s, 1H). ^{13}C -NMR (75 MHz, $CDCl_3$): δ 16.0, 28.5, 30.5, 31.5, 32.2, 36.5, 36.9, 41.0, 41.7, 69.8, 80.0, 156.8, 157.5, 172.8, 177.2, 177.8, 183.2, 183.4, 189.1, 189.4. ESI (+)-HRMS: m/z (%) calcd for $C_{14}H_{22}N_2NaO_5^+$ $[M+Na]^+$ 321.1421; found 321.1420.

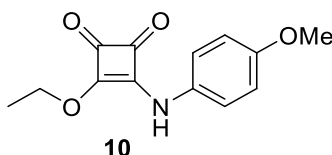
Then, the Boc-protected ethyl squaramate **1a** (120 mg, 0.40 mmol) was dissolved in dichloromethane (10 mL) followed by 157 μL of TFA (2.00 mmol). The reaction was stirred at room temperature for 8 h and the solvent was removed under rotary evaporation. The remaining TFA was removed by suspending the resulting oil in hexane ($\times 3$) yielding **1a** as a white solid, 122 mg, 97%. 1H -NMR (300 MHz, $DMSO-d_6$): δ 1.38 (t, $J = 7.0$ Hz, 2.7H), 1.79 (m, $J = 7.5$ Hz, 2H), 2.83 (m, $J = 6.5$ Hz, 2.2H), 3.35 (q, $J = 6.9$ Hz, 1.3H), 3.53 (q, $J = 7.2$ Hz, 1.5H), 4.66 (q, $J = 7.1$ Hz, 2.3H), 7.68 (br s, 2.8H), 8.62 (br s, 0.5H), 8.79 (br s, 0.5H). ^{13}C -NMR (75 MHz, $DMSO-d_6$): δ 16.2, 29.2, 36.9, 41.1, 69.9, 174.8, 186.0. ESI (+)-HRMS: m/z (%) calcd for $C_9H_{15}N_2O_3^+$ $[M+H]^+$ 199.1077; found 199.1077.



tert-butyl (3-((2-ethoxy-3,4-dioxocyclobut-1-en-1-yl) (methyl) amino) propyl)carbamate of 1b Analytical data are in accordance with those reported in literature.⁵

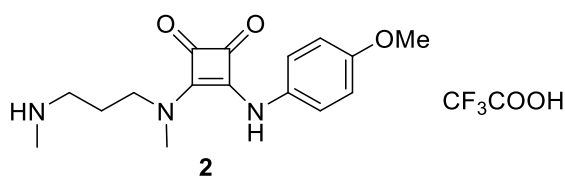
Silica-gel column chromatography (CH₂Cl₂:MeOH gradient, 1:0 to 20:1). Pale oil, 351 mg (yield 98 %). ¹H-NMR (300 MHz, DMSO-*d*₆): δ 1.47 (t, *J* = 4.8 Hz, 3H), 1.80 (m, 2H), 3.15 (t, *J* = 6.5 Hz, 2 H), 3.15 (s, 2H), 3.34 (s, 1H), 3.45 (t, *J* = 7.1 Hz, 0.8H), 3.73 (t, *J* = 6.5 Hz, 1.2H), 4.77 (q, *J* = 6.5 Hz, 2H), 5.06 (br s, 0.5H). ESI-HRMS (+) *m/z* (%): Calcd for C₁₅H₂₄N₂O₅Na⁺ [M+Na]⁺ 335.1577, found: 335.1560.

The corresponding trifluoroacetate salt was prepared analogously as for **1a** and used without further characterization



3-ethoxy-4-((4-methoxyphenyl)amino)cyclobut-3-ene-1,2-dione (10).

The ester was prepared according to a reported procedure.⁶ White yellow amorphous solid 384 mg (yield 58 %). ¹H-NMR (300 MHz, CDCl₃) δ 1.49 (t, *J* = 6.0 Hz, 3H), 2.96 (s, 6H), 4.85 (q, *J* = 6.0 Hz, 2H), 6.70 (d, *J* = 9.0 Hz, 2H), 7.14 (br s, 2H). ¹³C-NMR (75 MHz, DMSO-*d*₆) δ 188.11, 183.23, 177.83, 169.35, 156.23, 130.99, 121.36, 114.23, 69.37, 55.32, 15.67. ESI-HRMS(+) *m/z* (%): calcd C₁₃H₁₄NO₄⁺ [M+H]⁺ 248.0917; found 248.0914, 270.0734 [M+Na]⁺, 517.1585 [2M+Na]⁺.

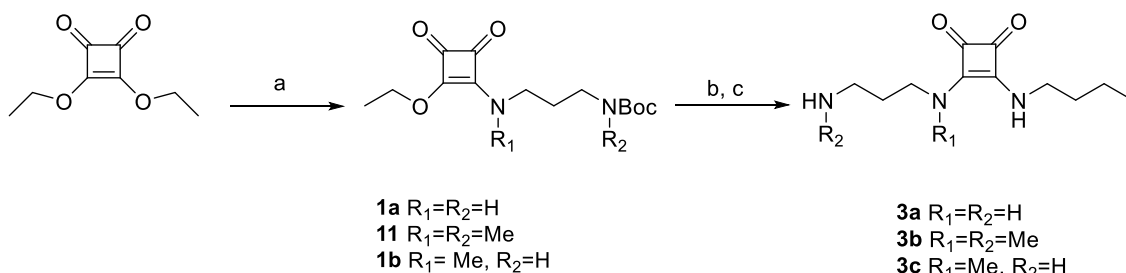


3-((2-((4-methoxyphenyl) amino)-3,4-dioxocyclobut-1-en-1-yl)(methyl) amino) -N-methylpropan-1-aminium trifluoroacetate (2).

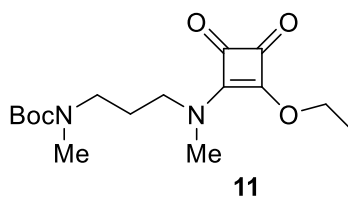
Squaramate ester **10** (1 equiv), **8** (1.2 equiv) and K₂CO₃ (2 equiv) were dissolved in 15 mL of EtOH and stirred at room temperature for 16 h. The crude solid was purified by column chromatography (SiO₂, CH₂Cl₂:MeOH, 95:5). The Boc-protected squaramide **2** was isolated as a white amorphous solid, 75 mg (yield 54 %). ¹H-NMR (300 MHz, DMSO-*d*₆) δ 1.37 (s, 9.4H), 1.80 (m, 2.1H), 2.77 (s, 3H), 3.19 (m, 5H), 3.63 (br m, 1.9H), 3.73 (s, 3.1H), 6.89 (d, *J* = 8.9 Hz, 2H), 7.16 (d, *J* = 8.9 Hz, 2.1H), 9.28 (s, 1H). ¹³C-NMR (75 MHz, DMSO-*d*₆, ppm) δ 25.5, 27.9, 33.7, 35.9, 48.3, 49.4, 52.9, 55.32, 114.23, 121.36, 114.23, 130.99, 156.1 168.7, 180.4, 184.5. ESI-HRMS (+) *m/z* (%): calcd C₂₁H₂₉N₃O₅Na⁺ [M+Na]⁺ 426.1999; found 426.2001.

The Boc-protected squaramide **2** was treated with TFA (10 equiv) in CH₂Cl₂ to afford the corresponding trifluoro acetic salt as a pale yellow oil, 40 mg (yield 79 %). ¹H-NMR (300 MHz, DMSO-*d*₆ ppm) δ 1.91 (m, 2.1H), 2.58 (s, 3.4H), 2.93 (br m, 2.1 H), 3.23 (s, 2.9 H), 3.74 (s + m, 4.8 H), 6.90 (d, *J* = 9.0 Hz, 2.1 H), 7.17 (d, *J* = 8.9 Hz 2.0 H), 8.35 (br s, 2.0 H), 9.34 (br s, 0.9 H). ¹³C-NMR (75 MHz, DMSO-*d*₆, ppm) δ: 24.0, 32.4, 45.4, 55.2, 113.7, 114.5, 122.5, 131.6, 155.8, 168.9, 180.5, 184.5. ESI-HRMS (+) *m/z* (%): calcd C₁₆H₂₂N₃O₃⁺ 304.1656; found 304.1654 [M+H]⁺.

Scheme S2. Synthesis of squaramido esters **1a**, **11** and **1b** and squaramides **3a-c**

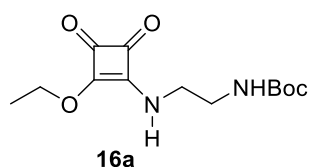


- a) BocR₂N(CH₂)₃NHR₁ in acetonitrile, r.t; b) CH₃-(CH₂)₃NH₂, in ethanol, r.t; c) TFA-CH₂Cl₂ or HCl



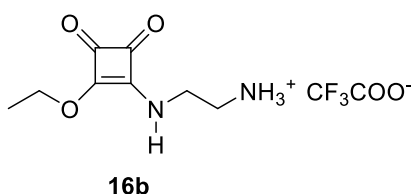
tert-butyl (3-((2-ethoxy-3,4-dioxocyclobut-1-en-1-yl) (methyl) amino) propyl) (methyl) carbamate, (**11**).

The corresponding *N*-Boc-diamine (1 equiv) was added dropwise to a solution of diethyl squarate (1.1 equiv) in CH₃CN (0.02 M). The solution was stirred overnight at room temperature. After reaction time, the solvent was removed and the crude was further purified either by Silica-gel column chromatography (CH₂Cl₂:MeOH gradient, 1:0 to 20:1). Pale oil, 379 mg (yield 90 %). ¹H-NMR (300 MHz, CDCl₃): δ 1.43 (s, 9.1H), 1.48 (t, *J* = 4.5, 3.2H), 1.81 (m, 2H), 2.87 (s, 3.1H), 3.17 (t, *J* = 6.5 Hz, 1.9H), 3.15 (s, 2H), 3.36 (s, 1.1H), 3.45 (t, *J* = 7.1 Hz, 0.9H), 3.73 (t, *J* = 6.5 Hz, 1.1H), 4.77 (q, *J* = 6.5 Hz, 2H), 5.06 (br s, 0.5H) ppm. ¹³C-NMR (150 MHz, CDCl₃): 15.4, 25.5, 27.9, 33.7, 35.9, 48.3, 49.4, 52.9, 69.1, 171.4, 175.7, 181.8, 181.9 ppm. ESI-HRMS (+) *m/z* (%): Calcd for C₁₆H₂₆N₂O₅Na⁺ [M+Na]⁺ 349.1734, found: 349.1734.



tert-butyl (2-((2-ethoxy-3,4-dioxocyclobut-1-en-1-yl)amino)ethyl)-carbamate (**16a**)

A solution of Boc-ethylenediamine (2.0 g, 12.5 mmol) in Et₂O (100 mL) was added dropwise to a stirred solution of diethyl squarate (2.55 g, 15.0 mmol) in Et₂O (20 mL). The mixture was stirred for 12 h at room temperature, and a white solid appeared. The solid was filtered, washed with cold Et₂O and dissolved in dichloromethane to afford **16a** (3.34 g, 11.7 mmol) as a white solid after the addition of pentane. Yield 94 %. Mp: 106 °C; ¹H-NMR (CDCl₃) δ: 6.83 (s, 0.6H), 6.06 (s, 0.4H), 4.97 (s, 1H), 4.75 (s, 2H), 3.76 (s, 0.8H), 3.55 (s, 1.2H), 3.36 (q, 2H, *J* = 4.5 Hz), 1.42 (s, 12H). ¹³C-NMR (CDCl₃) δ: 190.1, 183.1, 178.1, 173.2, 156.7, 80.2, 70.3, 45.6, 41.1, 28.8, 16.3. HRMS (MALDI-TOF): *m/z* calc. for C₁₃H₂₀N₂O₅K⁺: 323.1009, found 323.1009.

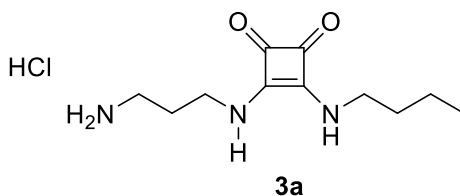


2-((2-ethoxy-3,4-dioxocyclobut-1-en-1-yl) amino)ethan-1-aminium-trifluoroacetate (**16b**).

Boc-protected ester **16a** (500 mg, 1.76 mmol) was dissolved in CH₂Cl₂ (20 mL) followed by 700 μl of TFA (8.80 mmol). The reaction was stirred at room temperature for 8 h and the solvent was removed under rotary evaporation. The remaining TFA was removed by suspending the resulting oil in hexane (× 3) yielding **16b** as a white amorphous solid, 514 mg, yield 98 %. ¹H-NMR (300 MHz, DMSO-*d*₆): δ = 1.37 (t, *J* = 6.9 Hz, 3H), 3.00 (m, *J* = 6 Hz, 2H), 3.52 (br, 0.9H), 3.70 (br, 1.1H), 4.66 (q, *J* = 6.9 Hz, 2H), 7.864 (br s, 3H), 8.576 (br s, 0.5H), 8.72 (br s, 0.5H). ¹³C-NMR (75 MHz, DMSO-*d*₆): δ = 15.6, 41.1, 41.5, 69.0, 172.1, 176.9, 182.4, 188.2. ESI (+)-HRMS: *m/z* (%) calcd for C₈H₁₃N₂O₃⁺ [M+H]⁺ 185.0921; found 185.0921.

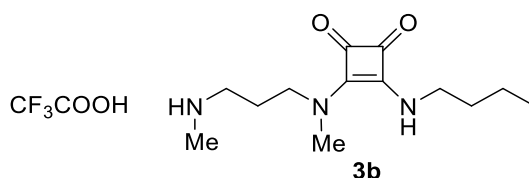
General procedure for synthesis of squaramides **3a-c**.

The corresponding squaramate **11a-c** (1 equiv) and *n*-butylamine (1.1 equiv) were dissolved in EtOH (0.05 M). The reaction mixture was stirred overnight at room temperature. After reaction time, the solvent was removed and further purified either by precipitation or by silica-gel chromatography. The resulting *N*-Boc protected compound was treated with HCl or trifluoroacetic acid to afford the final product as an ammonium salt.



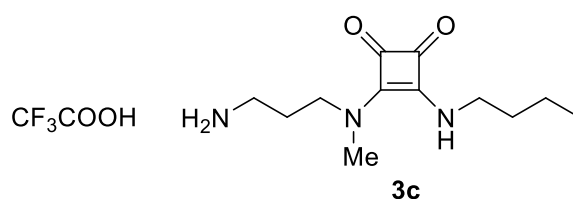
3-((2-(butylamino)-3,4-dioxocyclobut-1-en-1-yl) amino) propan-1-aminium (**3a**).

Grey amorphous solid. 27 mg (yield 88 %). ¹H-NMR (300 MHz, D₂O): δ 0.91 (t, *J* = 7.4 Hz, 3.1H), 1.37 (m, *J* = 7.5, 2.1H), 1.60 (m, *J* = 7.1, 2.0), 2.02 (m, *J* = 7.1, 2.0), 3.09 (t, *J* = 7.6 Hz, 2.1), 3.61 (br s, 1.9H), 3.71 (br s, 1.9H). ¹³C-NMR (150 MHz, D₂O): δ 13.0, 19.0, 28.4, 32.4, 36.7, 41.1, 44.2, 168.2, 168.5, 181.2, 182.0. ESI-HRMS (+) *m/z* (%): Calcd for C₁₁H₂₀N₃O₂⁺ [M+H]⁺ 226.1539, found: 226.1542.



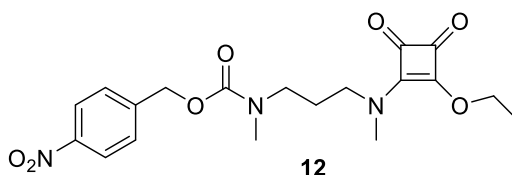
3-(butylamino)-4-(methyl(3-(methylamino)propyl)amino)cyclobut-3-ene-1,2-dione trifluoroacetic salt. (3b). Analytical data are in accordance with those reported in literature.⁷

Silica-gel column chromatography (CH₂Cl₂: MeOH gradient, 40:1 to 20:1). Pale oil, 76 mg (yield 72 %). ¹H-NMR (300 MHz, CDCl₃): δ 0.94 (t, *J* = 7.2 Hz, 3.1H), 1.38 (m, *J* = 7.3 Hz, 2.1H), 1.43 (s, 9.1H), 1.62 (m, 2H), 1.81 (m, 2H), 2.87 (s, 3.1H), 3.17 (t, *J* = 6.5, 1.9H), 3.15 (s, 2H), 3.36 (s, 1.1H), 3.45 (t, *J* = 7.1, 0.9H), 3.73 (t, *J* = 6.5, 1.1H), 4.77 (q, *J* = 6.5, 2H), 5.06 (br s, 0.5H). ¹³C NMR (150 MHz, D₂O): δ 13.6, 19.1, 23.4, 23.9, 32.2, 33.1, 43.0, 45.3, 54.4, 167.0, 167.4, 181.9, 182.2. ESI-HRMS (+) *m/z* (%): Calcd for C₁₈H₃₁N₃O₄Na⁺ [M+Na]⁺ 376.2207, found: 376.2207.



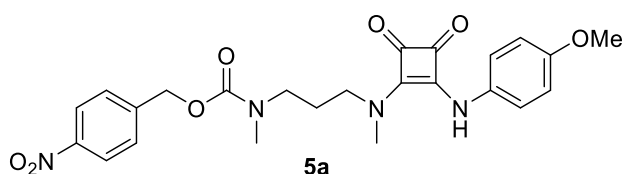
3-((2-(butylamino)-3,4-dioxocyclobut-1-en-1-yl)(methyl)amino)propan-1-aminiumtrifluoroacetate, (3c).

Silica-gel column chromatography (CH₂Cl₂: MeOH gradient, 40:1 to 20:1). Pale oil, 378 mg (yield 97%). ¹H NMR (600 MHz, CDCl₃): δ 0.95 (t, *J* = 7.3 Hz, 3H), 1.38 (m, *J* = 7.6 Hz, 2H), 1.44 (s, 2 H), 1.62 (m, 4H), 1.84 (m, *J* = 6.6 Hz, 2H), 3.16 (q, *J* = 6.2 Hz, 2H), 3.24 (s, 3 H), 3.57 (t, *J* = 7.8 Hz, 2H), 3.74 (q, *J* = 6.8 Hz, 2H), 5.10 (s, 0.5H), 5.66 (s, 0.5 H). ¹³C NMR (75 MHz, DMSO-*d*₆): δ 13.6, 19.1, 25.7, 33.1, 36.3, 43.2, 48.1, 167.5, 181.8, 182.4. ESI-HRMS (+) *m/z* (%): Calcd for C₁₇H₂₉N₃O₄Na⁺ [M+Na]⁺ 362.2050, found: 362.2051.

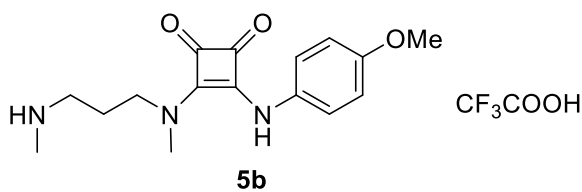


4-nitrobenzyl 3-(2-ethoxy-3,4-dioxocyclobut-1-en-1-yl)(methyl)amino propyl(methyl) carbamate. (12).

677 mg (2.41 mmol) of protected amine **9**, 491 mg (2.89 mmol) of ethyl squarate and 0.4 mL (2.89 mmol) of Et₃N were dissolved in Et₂O (15 mL) and the reaction was stirred for 16 h at room temperature. After the reaction time, the solvent was removed by rotary evaporation and the resulting crude residue was purified by silica-gel column chromatography (CH₂Cl₂:MeOH 40:1 v/v) to afford **12** as pale oil. 782 mg (yield 80 %). ¹H-NMR (300 MHz, CDCl₃) δ: 1.45 (m, *J* = 6.7 Hz, 3.7 H), 1.90 (m, *J* = 7.3 Hz, 2H), 2.98 (s, 2.7H), 3.10 (m, 1.1H), 3.16 (s, 1.1H), 3.35 (s, 2.9H), 3.43 (t, *J* = 8.0Hz, 1H), 3.72 (t, *J* = 7.0 Hz, 1.3H), 4.76 (q, *J* = 7.0Hz, 2.1H), 5.2 (s, 2H), 7.52 (d, *J* = 8.9 Hz, 1.5H), 8.23 (dd, *J* = 8.8Hz, 1.5H) ppm. ¹³C-NMR (150 MHz, CDCl₃) 15.3, 24.9, 29.19, 33.5, 35.8, 65.3, 69.2, 123.3, 127.5, 143.7, 154.9, 171.6, 171.8, 175.7, 181.92, 188.2 ppm. ESI-HRMS (+) *m/z* (%): calcd for C₁₉H₂₄N₃O₇⁺ [M+H]⁺ 406.1609; found 406.1607.



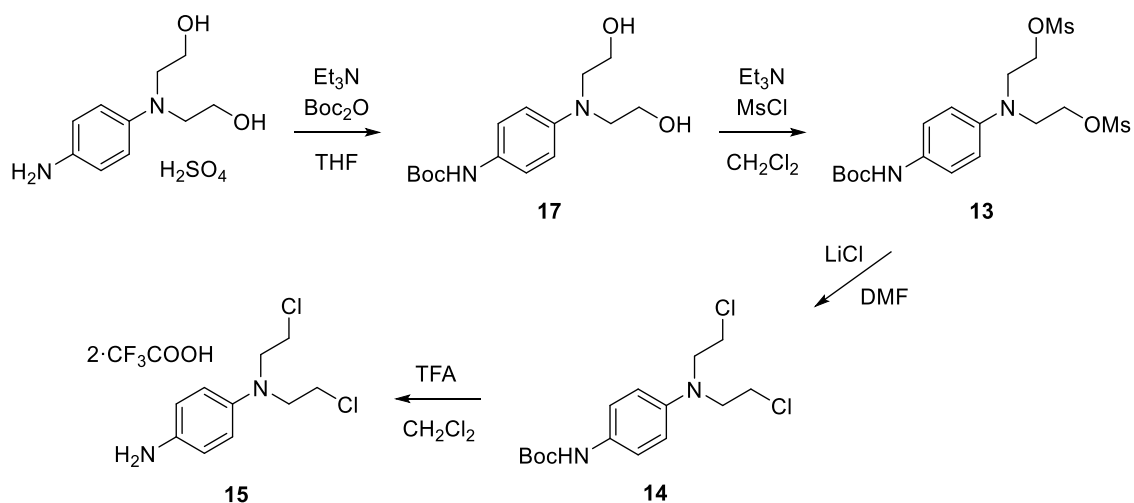
4-nitrobenzyl (3-((2-((4-methoxyphenyl)amino)-3,4-dioxocyclobut-1-en-1-yl)(methyl)amino)propyl)(methyl)carbamate (5a). 30 mg (0.11 mmol) of **12** and 26 mg of *p*-methoxyaniline (0.21 mmol) were mixed in 5 mL of EtOH and stirred at 50 °C for 16 h. After the reaction time, the solvent was removed under rotary evaporation and the crude solid was purified by column chromatography (SiO₂, CH₂Cl₂:MeOH, 98:2) to afford **5a** as a brown solid, 30 mg (yield 59 %). ¹H-NMR (300 MHz, CDCl₃) δ: 1.93 (m, *J* = 6.7 Hz, 2.0 H), 2.99 (s, 2.8 H), 3.11 (s, 2.6 H), 3.36 (t, *J* = 6.9 Hz, 2.0 H), 3.63 (t, *J* = 7.1 Hz, 2.1 H), 3.79 (s, 2.9 H), 5.18 (1.9 H), 6.86 (d, *J* = 8.9 Hz, 1.8 H), 7.16 (d, *J* = 8.6 Hz, 2.1 H), 7.48 (d, *J* = 8.7 Hz, 1.5 H), 8.20 (d, *J* = 8.7 Hz, 1.4 H). ¹³C-NMR (75 MHz, CDCl₃, ppm) δ: 26.2, 34.7, 37.6, 46.4, 49.9, 55.6, 66.0, 114.5, 122.6, 123.9, 128.1, 131.6, 144.1, 147.7, 156.2, 157.1, 164.3, 169.6, 182.1, 184.7. ESI-HRMS (+) *m/z* (%): calcd for C₂₄H₂₇N₄O₇⁺ [M+H]⁺ 483.1874; found 483.1876 [M+H]⁺, 505, 1695 [M+Na]⁺.



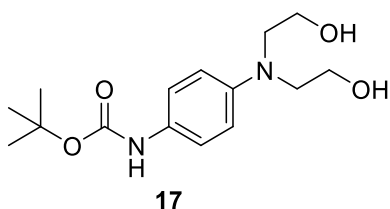
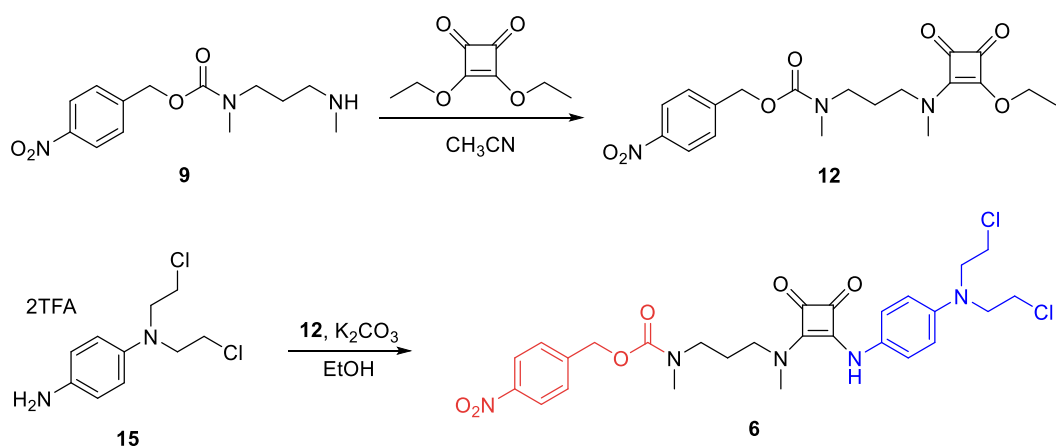
(3-((2-((4-methoxyphenyl)amino)-3,4-dioxocyclobut-1-en-1-yl)(methyl)amino)-N-methylpropan-1-aminium trifluoroacetate (5b)

5a was treated with with TFA (10 equiv) in CH₂Cl₂ to afford 40 mg (yield 79 %) of **5b** as a pale yellow oil. ¹H NMR (300 MHz, DMSO-*d*₆) δ 1.91 (m, 2.1H), 2.58 (s, 3.4H), 2.93 (br m, 2.1H), 3.23 (s, 2.9H), 3.74 (s + m, 4.8H), 6.90 (d, *J* = 9.0 Hz, 2.1H), 7.17 (d, *J* = 8.9 Hz 2.0H), 8.35 (br s, 2.0H), 9.34 (br s, 0.9H). ¹³C NMR (75 MHz, DMSO-*d*₆, ppm) δ 24.0, 32.4, 45.4, 55.2, 113.7, 114.5, 122.5, 131.6, 155.8, 168.9, 180.5, 184.5. ESI-HRMS (+) *m/z* (%): calcd C₁₆H₂₂N₃O₃⁺ 304.1656; found 304.1654 [M+H]⁺.

Scheme S3. Four-step synthesis of parent nitrogen mustard **15**



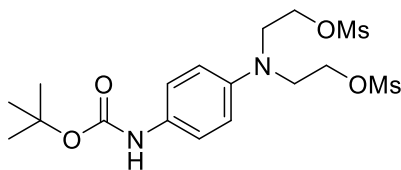
Scheme S4. Synthesis of self-immolative prodrug **6**



***tert*-butyl (4-(bis(2-hydroxyethyl)amino)phenyl)carbamate (**17**).**

682 mg (2.32 mmol) of *N,N*-bis(2-hydroxyethyl)-1,4-phenylenediamine and 1 mL (6.95 mmol) of Et_3N were dissolved in CH_2Cl_2 (20 mL). The reaction was stirred for 1 h to achieve the complete deprotonation of amine starting material. Then, 632 mg (2.90 mmol) of Boc_2O dissolved in CH_2Cl_2 were added and the reaction was stirred at room temperature for 8 h. After the reaction time, the solvent was removed under rotary evaporation and the crude residue was purified by silica-gel column chromatography (CH_2Cl_2 : MeOH 30:1 v/v) to afford **17** as an amorphous white solid. 578

mg (yield 84 %). ¹H-NMR (300 MHz, CDCl₃) δ: 1.44 (s, 9.1H), 3.49 (q, *J* = 5.9 Hz, 4.4H), 4.71 (t, *J* = 5.6 Hz, 2.1H), 6.57 (d, *J* = 9.4 Hz, 2.1H), 7.18 (d, *J* = 9.6 Hz, 2H), 8.85 (br s, 0.9H).

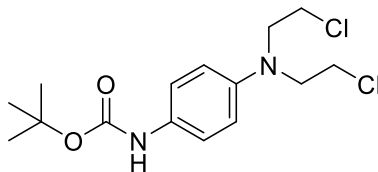


13

((4-((*tert*-butoxycarbonyl)amino)phenyl)azanediyl)bis(ethane-2,1-diyl)dimethane-sulfonate (13).

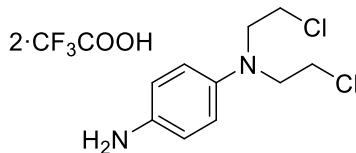
Analytical data are in accordance with those reported in literature.⁸

578 mg (1.95 mmol) of **12** and 0.82 mL (5.85 mmol) of distilled Et₃N were dissolved in anhydrous CH₂Cl₂ (15 mL) and cooled to 0°C in an ice bath under argon atmosphere. Then, 0.41 mL (5.27 mmol) of MsCl were added dropwise and the reaction was stirred for 0.5 h at 0°C. Then, the reaction was quenched with 50 mL of NaHCO₃ 5 % and the product was extracted with CH₂Cl₂ (3 × 10 mL). The organic phase was dried with brine (2 × 10 mL) and Na₂SO₄ and concentrated under rotary evaporation to afford **13** as pale yellow oil. 682 mg (yield 77 %). ¹H-NMR (300 MHz, CDCl₃) δ: 1.45 (s, 8.2H), 3.14 (s, 5.9H), 3.66 (t, *J* = 5.8 Hz, 4H), 4.27 (t, *J* = 5.6 Hz, 3.8H), 6.71 (d, *J* = 9.6 Hz, 2.2H), 7.27 (d, *J* = 8.2 Hz, 2H), 8.98 (br s, 1.2H).



14

tert-butyl (4-(bis(2-chloroethyl)amino)phenyl)carbamate (14). 682 mg (1.51 mmol) of dimesylate **13** and 192 mg (4.52 mmol) of LiCl were dissolved in 2 mL of anhydrous DMF and heated to 117 °C under argon atmosphere. After 0.5 h the reaction was cooled down to 0 °C in an ice-bath and 10 mL of HCl 3N were added. The product was extracted with CH₂Cl₂ (3 × 10 mL) and the organic phase was dried with brine (2 × 10 mL) and Na₂SO₄. The solvent was removed under rotary evaporation and the crude was digested with hexane to afford **14** as an amorphous white solid. 371 mg (yield 74 %). ¹H-NMR (300 MHz, CDCl₃) δ: 1.45 (s, 8.7H), 3.67 (m, 8H), 6.66 (d, *J* = 9.1 Hz, 2H), 7.26 (d, *J* = 8.3 Hz, 1.8H), 8.97 (br s, 0.9H).



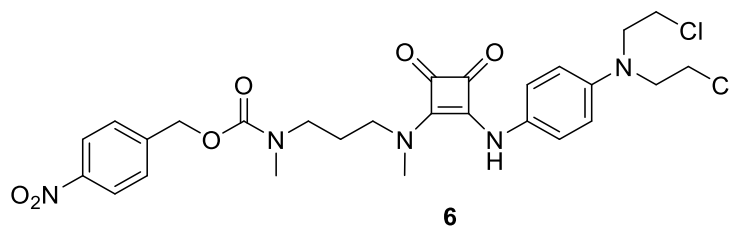
15

***N'*,*N'*-bis(2-chloroethyl)benzene-1,4-diaminium trifluoroacetate (15).**

Analytical data are in accordance with those reported in literature.⁸

371 mg (1.11 mmol) of boc-protected compound **14** were dissolved in CH₂Cl₂ (5 mL) and 0.86 mL (11 mmol) of TFA were added and the reaction was stirred at 30 °C for 48 h. After the reaction time the solvent was removed by rotary evaporation and the resulting oil crude was digested with

hexane to afford **15** as a brown amorphous solid. 219 mg (43 % yield). $^1\text{H-NMR}$ (300 MHz, CDCl_3) δ : 3.72 (s, 7.4H), 6.83 (d, $J = 8.9$ Hz, 2.1H), 7.15 (d, $J = 8.9$ Hz, 2H), 9.50 (br s, 3.1H).



4-nitrobenzyl (3-((2-((4-bis(2-chloroethyl)amino)phenyl)amino)-3,4-dioxocyclobut-1-en-1-yl)(methyl)amino)propyl(methyl)carbamate (6**)**. 34 mg (0.08 mmol) of ester **12** were dissolved in EtOH (5 mL) and 25 mg (0.18 mmol) of K_2CO_3 were added and the suspension was stirred at room temperature. Then 46 mg of **15** (0.10 mmol) dissolved in EtOH (5 mL) were added dropwise and the reaction was stirred at room temperature for 16 h. After the reaction time, the solvent was removed by rotary evaporation and the resulting crude was purified by silica-gel column chromatography (CH_2Cl_2 :MeOH 20:1 v/v) to afford **6** as an amorphous brown solid, 12 mg (yield 24 %). $^1\text{H-NMR}$ (600 MHz, CDCl_3) δ : 1.93 (s, 2H), 2.98 (s, 3.2H), 3.14 (s, 2.7H), 3.36 (t, $J = 6.9$ Hz, 2H), 3.60 (t, $J = 6.9$ Hz, 4.6H), 3.64 (s, 1.4H), 3.69 (t, $J = 7.0$ Hz, 4.2H), 5.18 (s, 2H), 6.62 (d, $J = 8.8$ Hz, 2H), 7.12 (s, 2H), 7.48 (d, $J = 8.2$ Hz, 2.4H), 7.56 (br s, 0.4H) 8.19 (d, $J = 8.2$ Hz, 2H). $^{13}\text{C-NMR}$ (150 MHz, CDCl_3) δ : 29.8, 34.7, 37.6, 40.6, 46.5, 49.9, 53.7, 66.0, 112.7, 122.9, 123.1, 123.9, 128.1, 129.1, 143.8, 144.1, 147.7, 164.3, 169.4, 182.1, 184.6. ESI-HRMS (+) m/z (%): calcd for $\text{C}_{27}\text{H}_{31}\text{N}_5\text{O}_6\text{Cl}_2\text{Na}^+$ $[\text{M}+\text{Na}]^+$ 614.1544; found 614.1546.

3. ^1H and ^{13}C NMR spectra of the new compounds

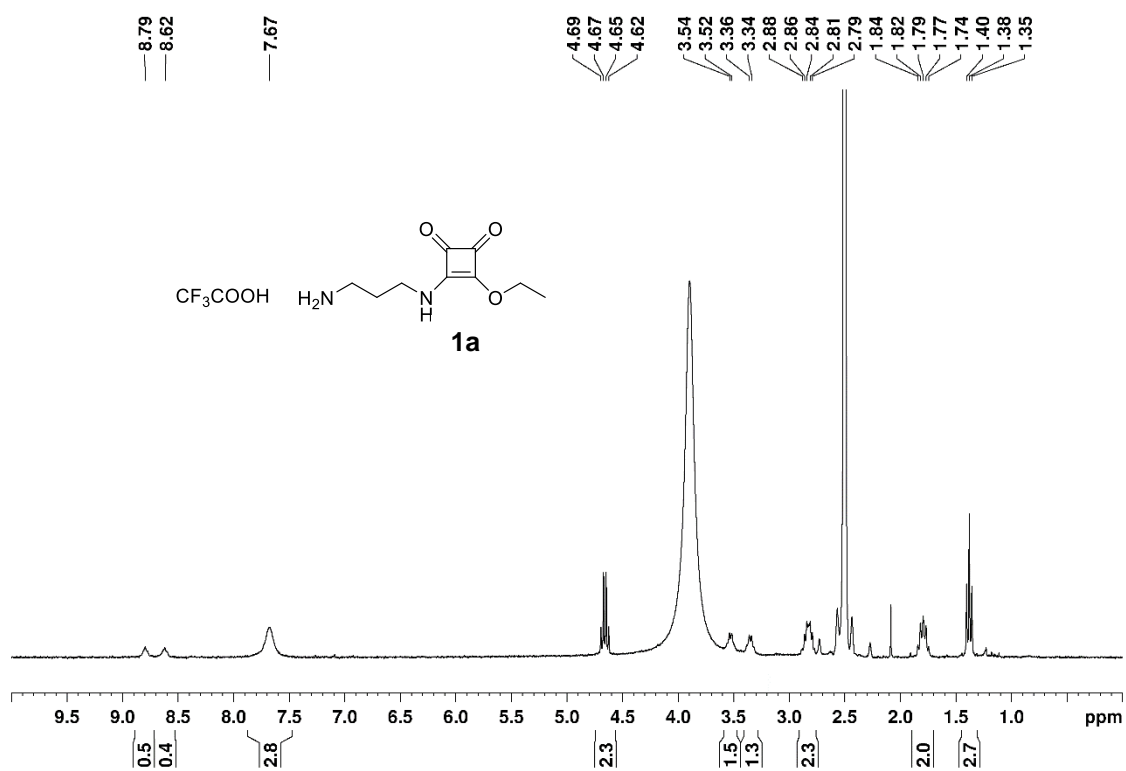


Figure S1. ^1H -NMR spectrum of **1a** in $\text{DMSO-}d_6$, 300 MHz, 298 K.

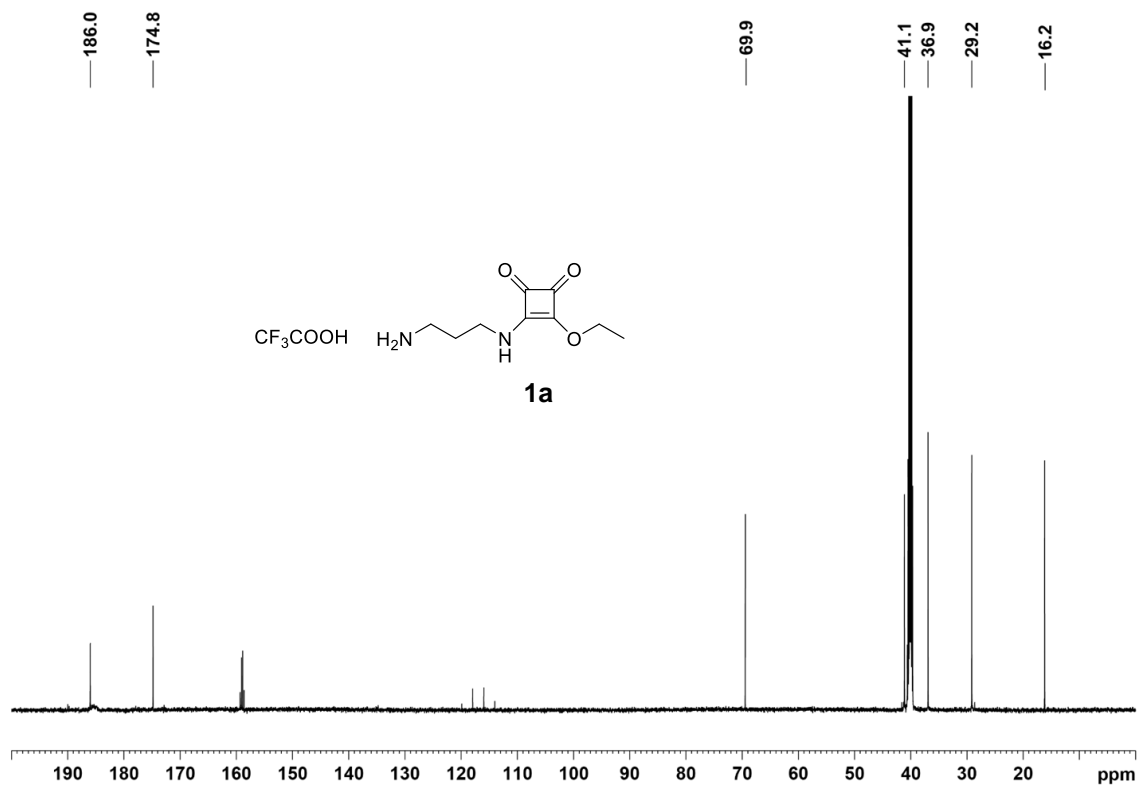


Figure S2. ^{13}C NMR spectrum of **1a** in $\text{DMSO-}d_6$, 300 MHz, 298 K.

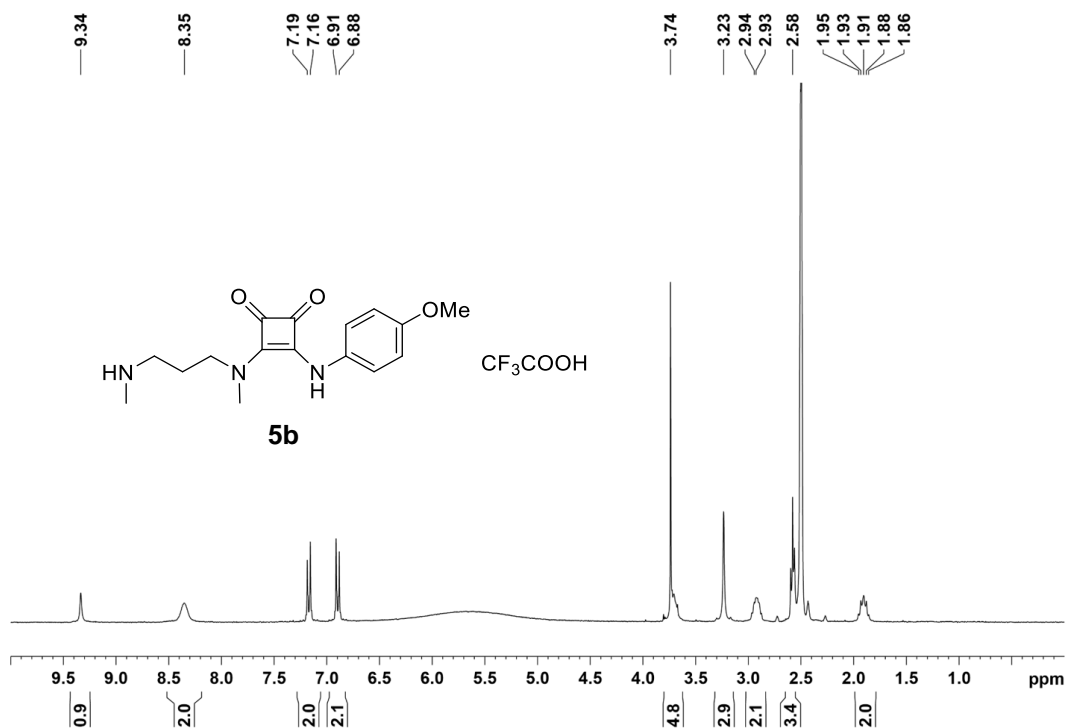


Figure S3. $^1\text{H-NMR}$ spectrum of **5b** $\text{DMSO-}d_6$, 300 MHz, 298 K.

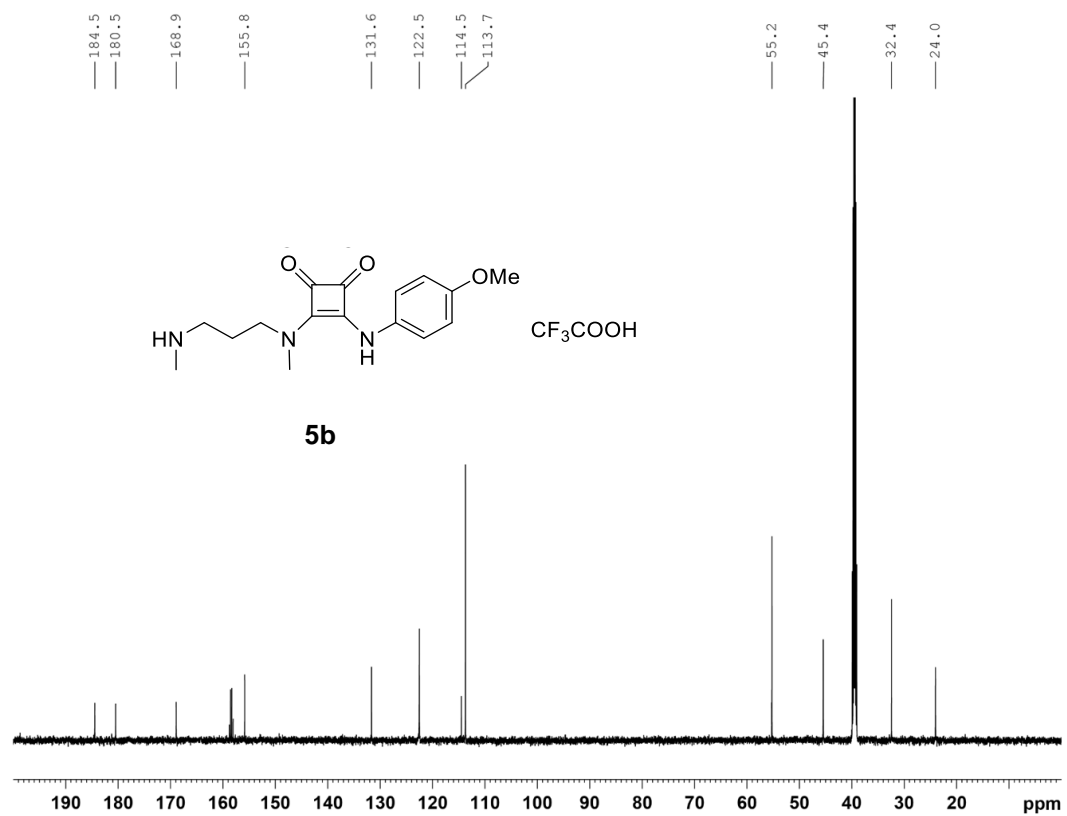


Figure S4. $^{13}\text{C-NMR}$ spectrum of **5b** DMSO , 75 MHz, 298 K.

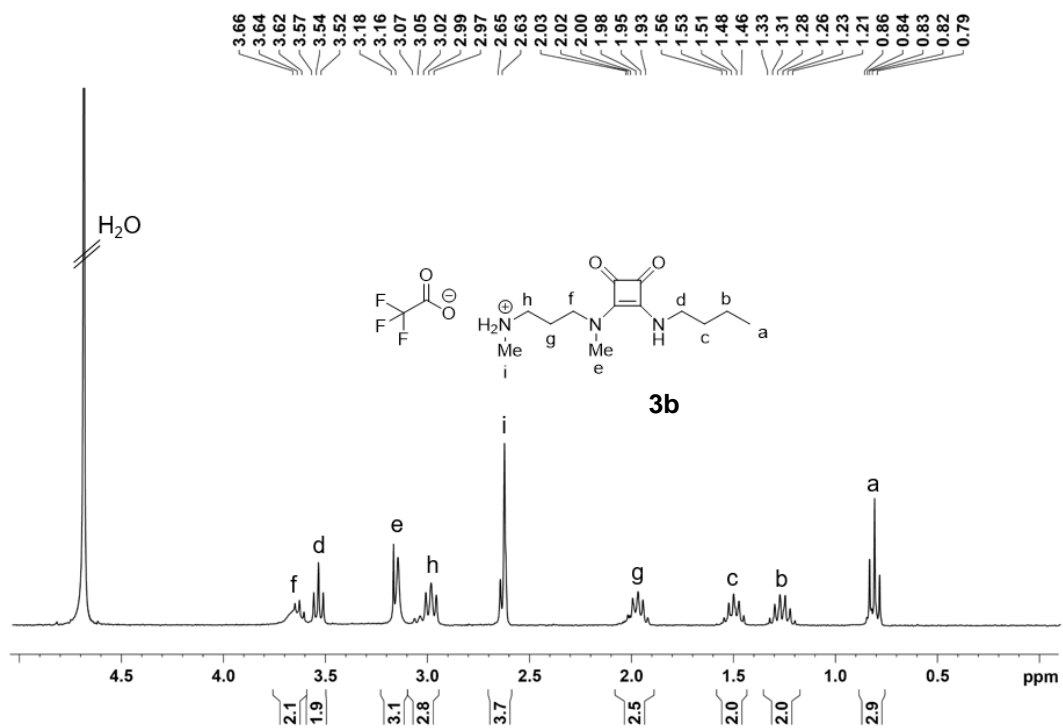


Figure S5. $^1\text{H-NMR}$ of **3b** D_2O , 300 MHz, 298 K, and the corresponding signal assignment.

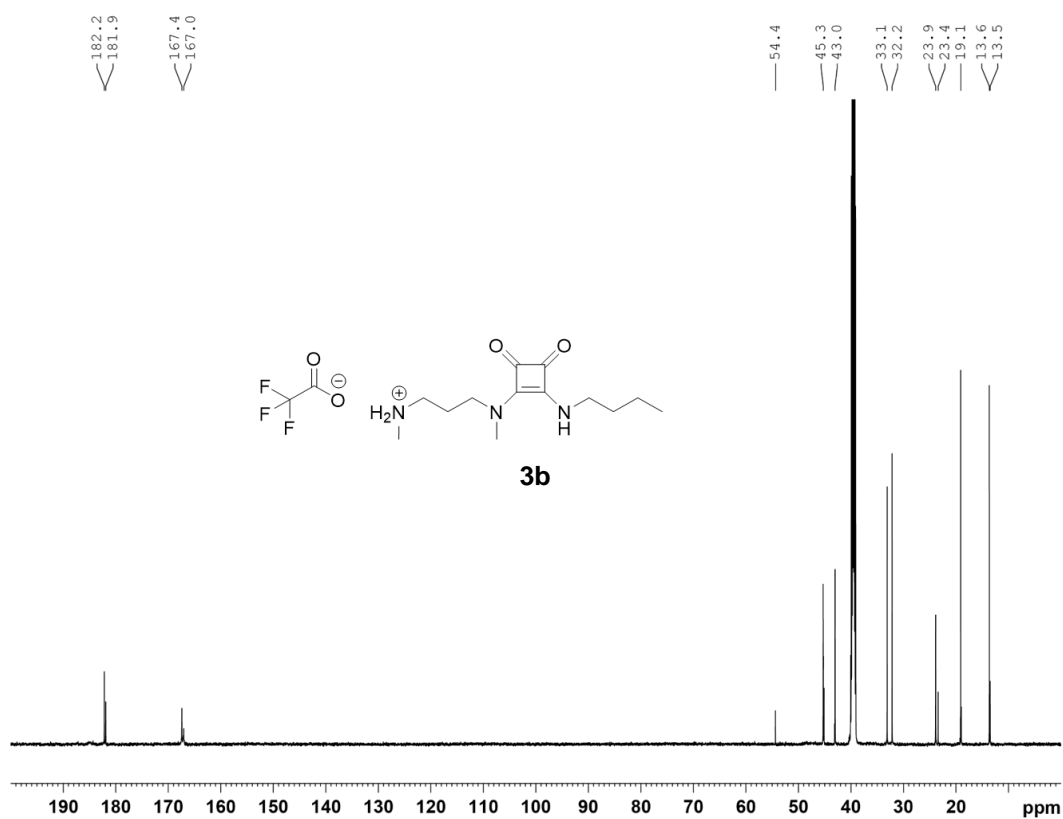
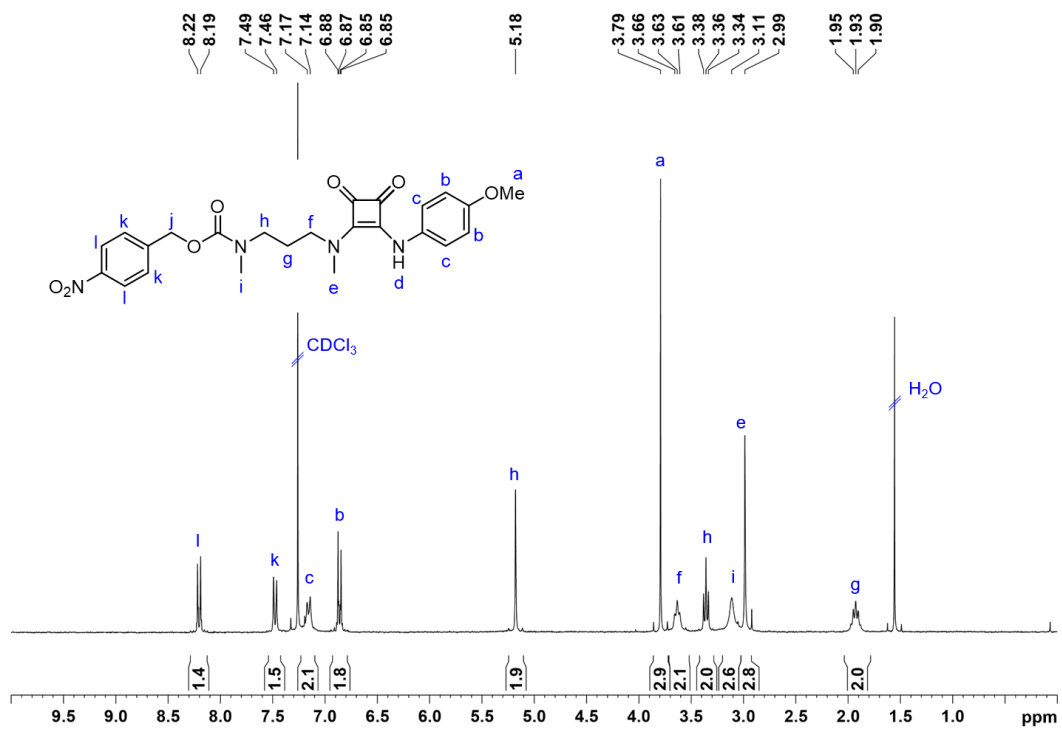
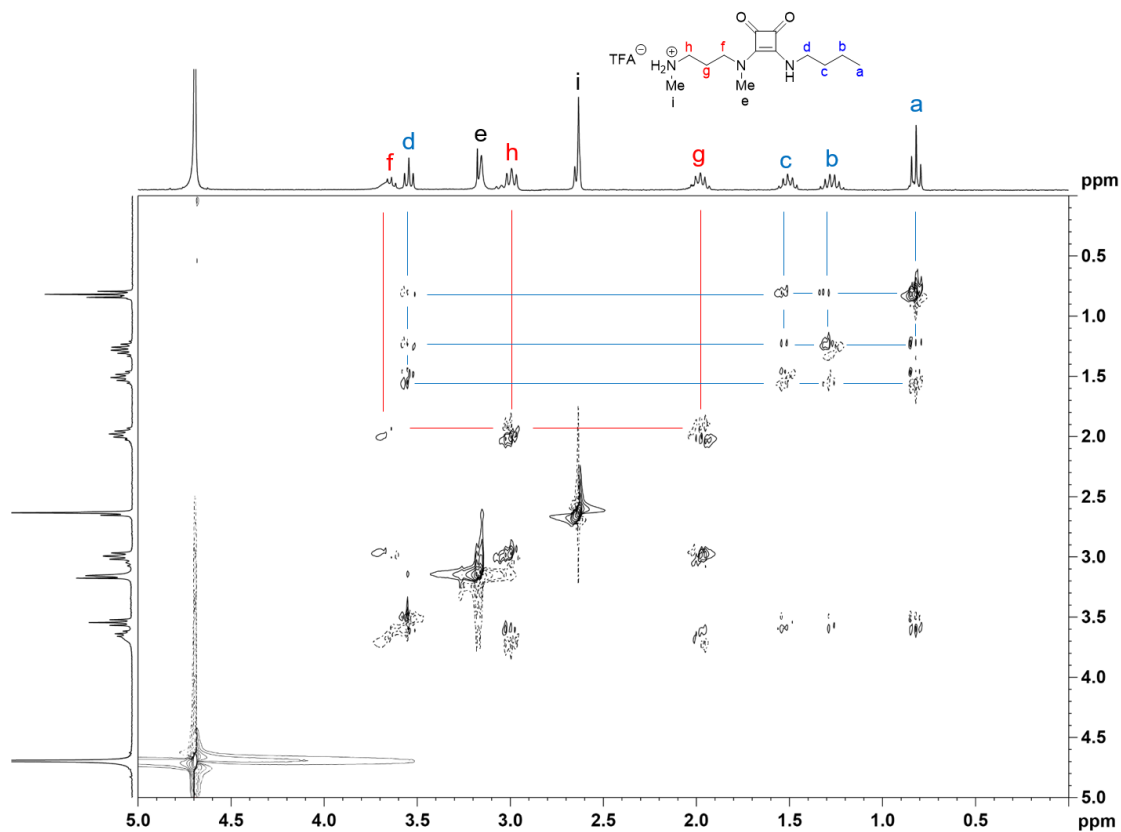


Figure S6. $^{13}\text{C-NMR}$ spectrum of **3b** in $\text{DMSO-}d_6$, 150 MHz, 298 K.



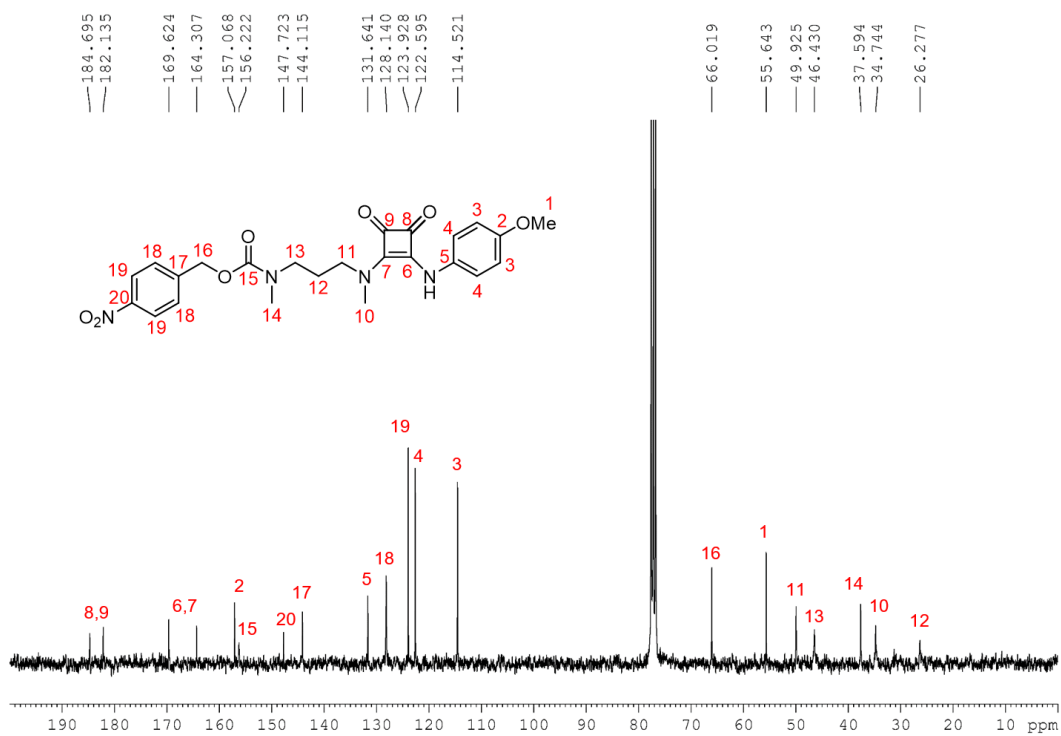


Figure S9. ^{13}C NMR spectrum of **5a** CDCl_3 , 75 MHz, 298 K and the corresponding signal assignment.

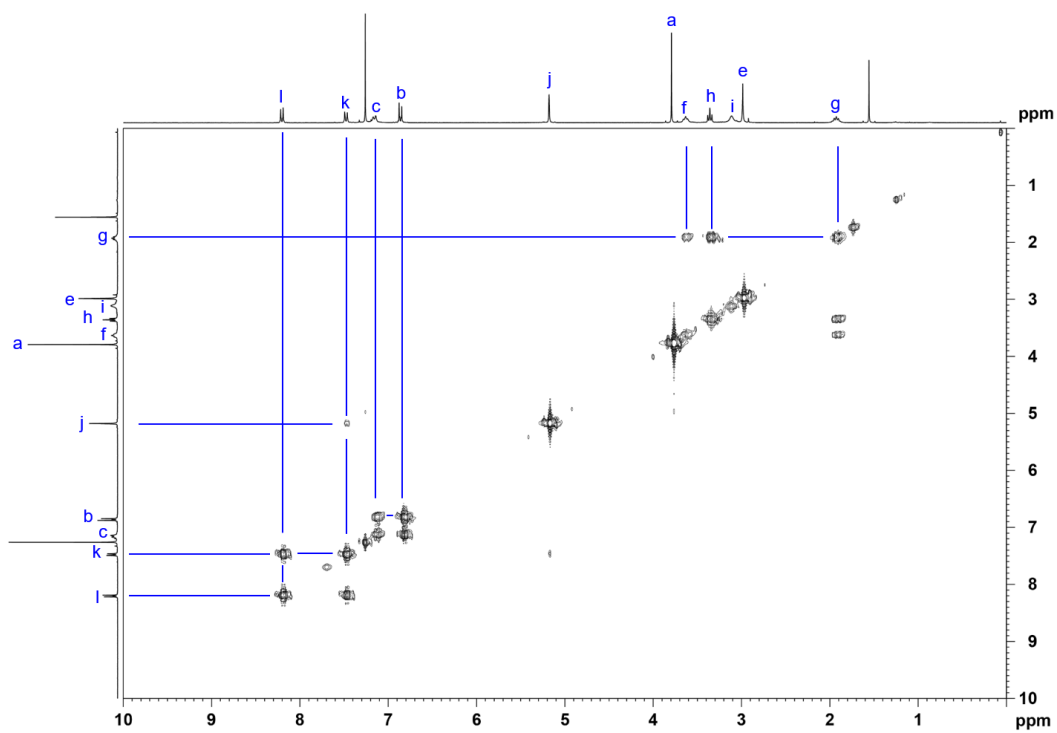


Figure S10. ^1H - ^1H COSY of compound **5a** CDCl_3 , 75 MHz, 298 K.

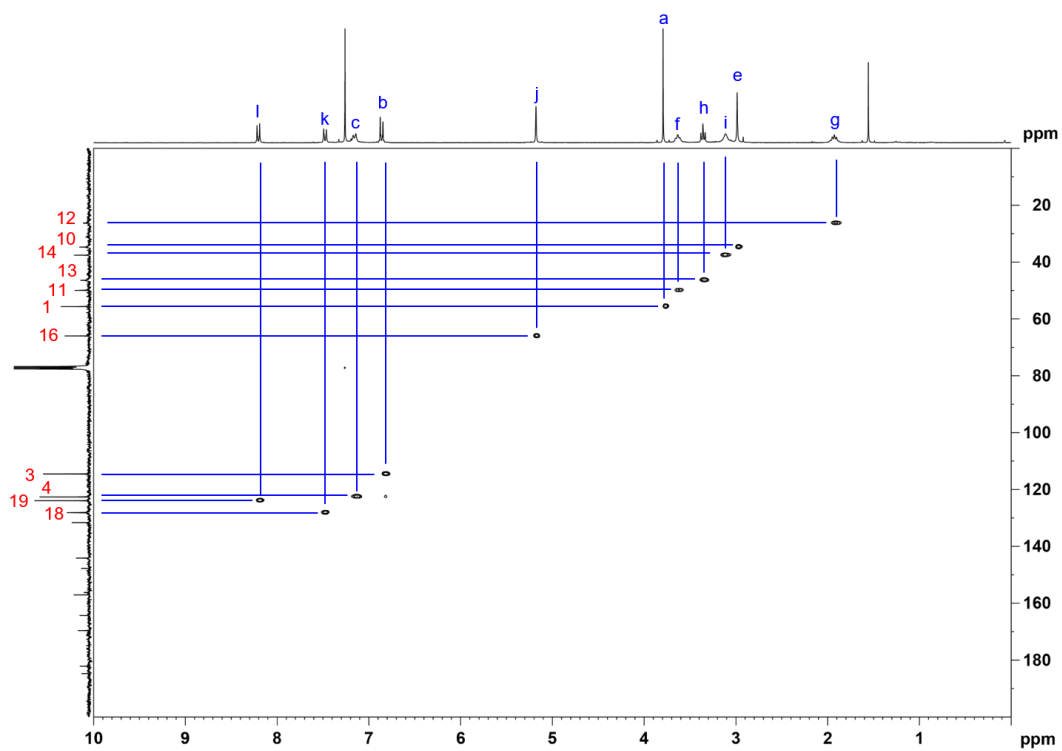


Figure S11. ^1H - ^{13}C HSQC of compound **5a** CDCl_3 , 75 MHz, 298 K.

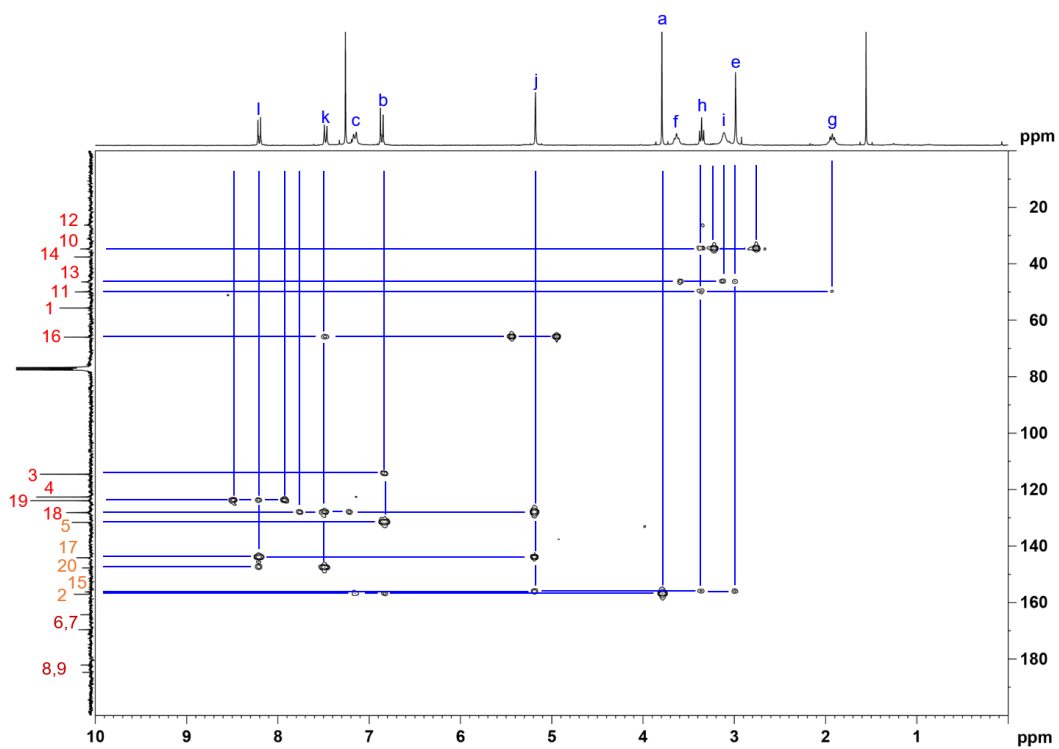


Figure S12. ^1H - ^{13}C HMBC of compound **5a** CDCl_3 , 75, 298 K.

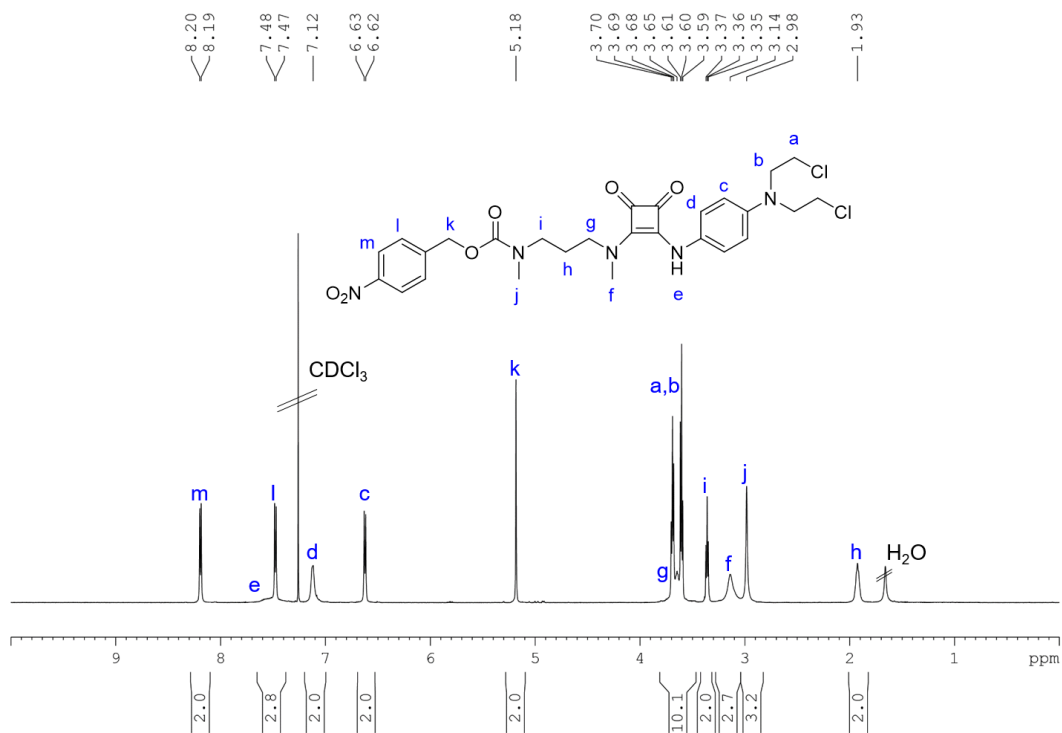


Figure S13. $^1\text{H-NMR}$ spectrum of compound 6 CDCl_3 , 600 MHz, 298 K, and the corresponding signal assignment.

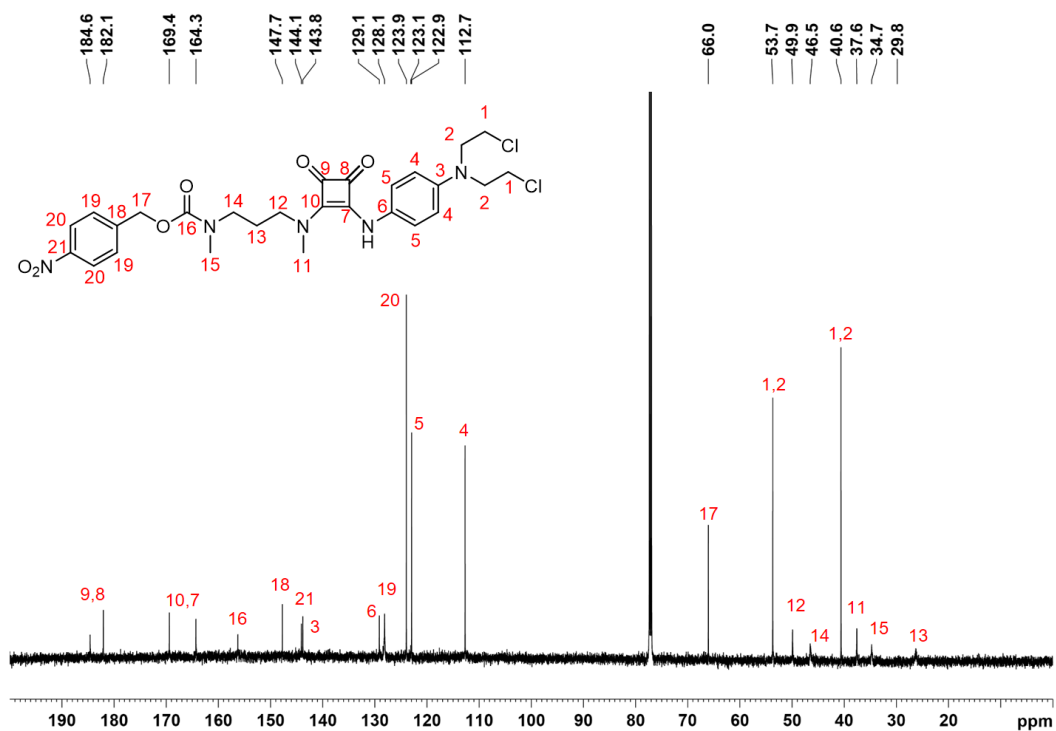


Figure S14. $^{13}\text{C-NMR}$ spectrum of compound 6 CDCl_3 , 150 MHz, 298 K, and the corresponding signal assignment.

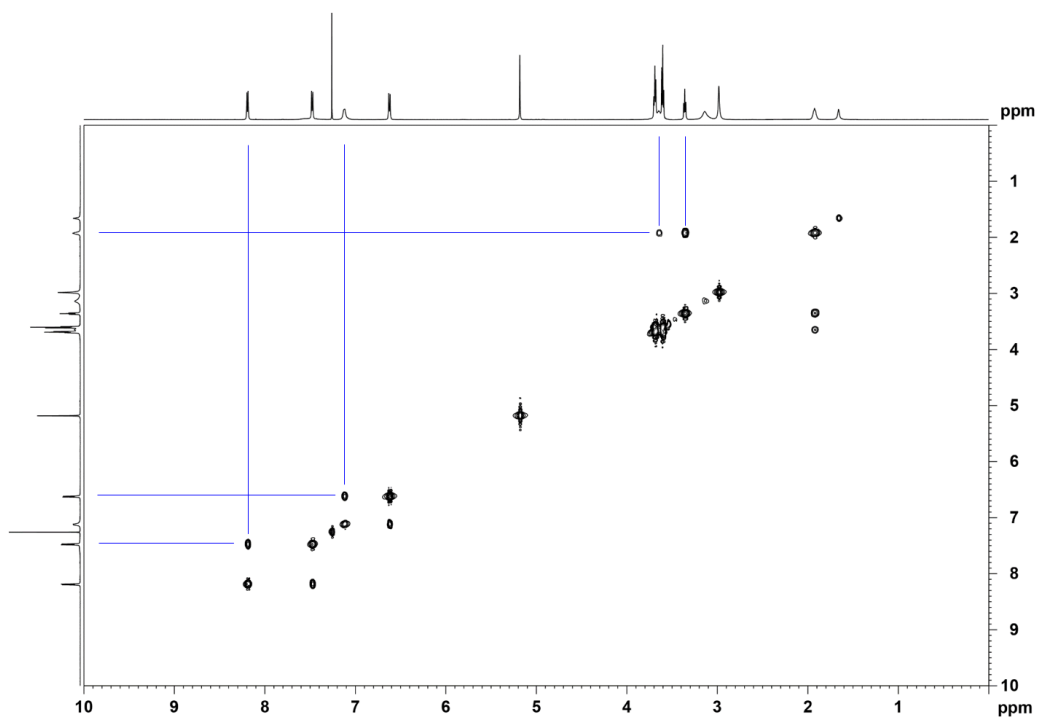


Figure S15. ^1H - ^1H COSY of compound **6** CDCl_3 , 150 MHz, 298 K.

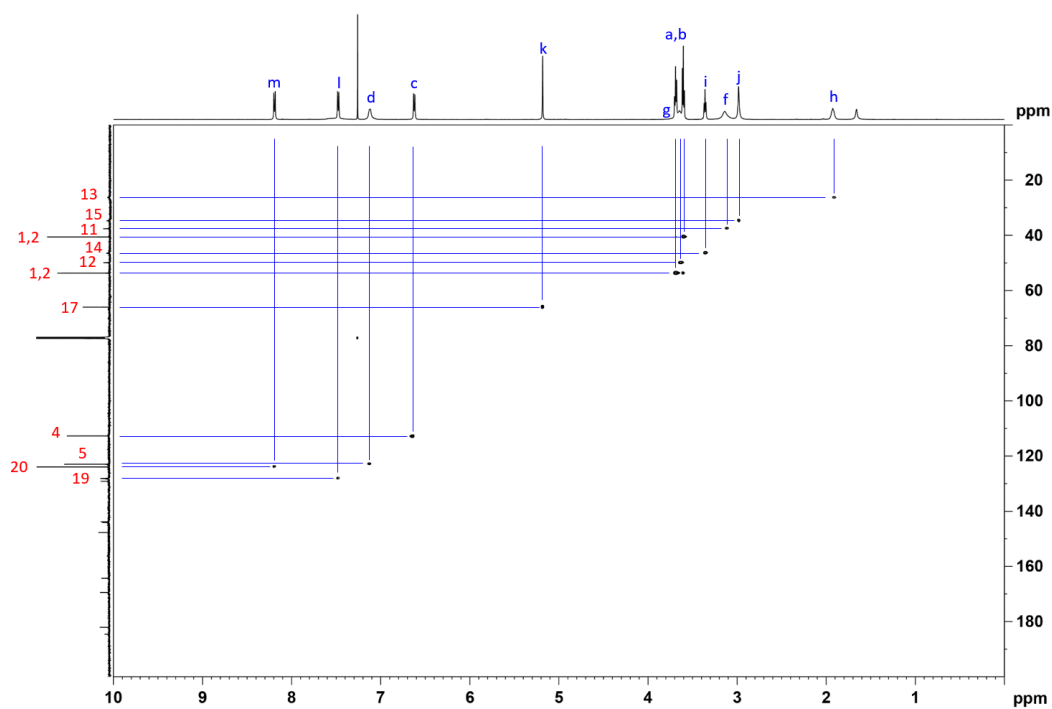


Figure S16. ^1H - ^{13}C HSQC of compound **6** CDCl_3 , 150 MHz, 298 K.

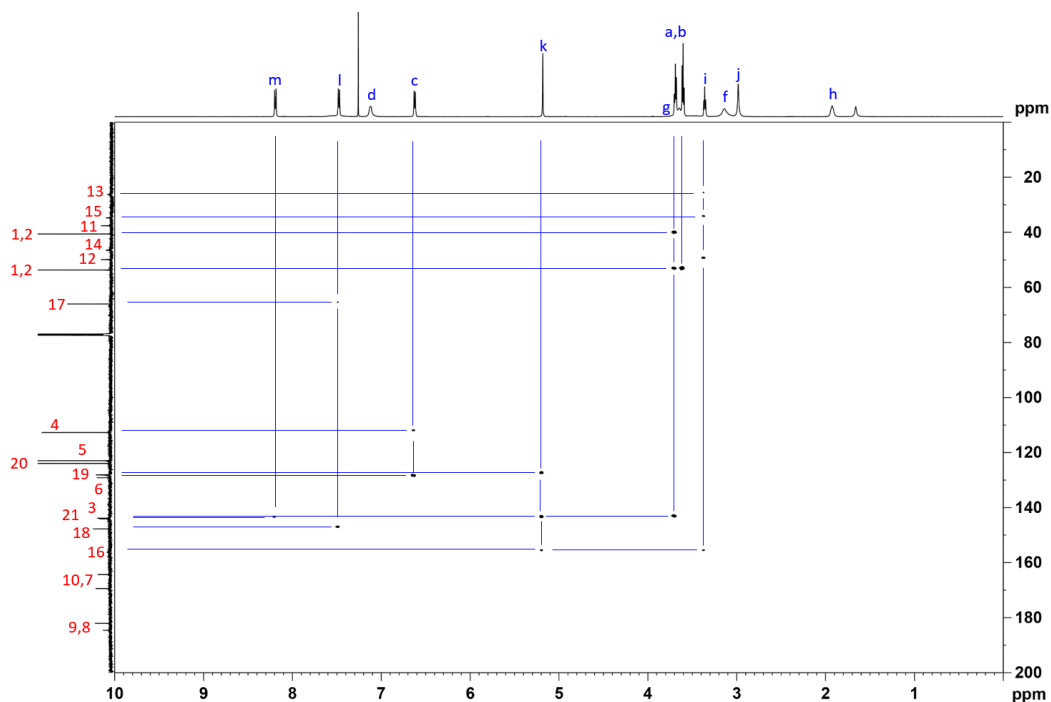


Figure S17. ^1H - ^{13}C HMBC of compound **6** CDCl_3 , 150 MHz, 298 K.

4. Self-immolative reactions kinetic studies

Formation of six-membered ring vs seven-membered ring cyclic squaramide

We have compared the cyclization reaction of compound **16b** vs. compound **1a** to optimize the alkyl length of the self-immolative spacer. In these studies, we confirmed that the cyclisation step to give a six-membered ring cyclic squaramide is less favoured than forming the corresponding seven-membered ring. At $\text{pH} \geq 7$, the ester group hydrolysis competes with the cyclization reaction, dropping, even more, the cyclization reaction yield. In our experiments with **16b**, we could not detect the formation of the corresponding cyclic compound, a pH ranging from 7 to 11, but the corresponding squaramic acid.

Figure S18 shows the changes observed in the UV spectrum of **16b** ($30 \mu\text{M}$, $\text{pH} 7$ at 37°C). In the figure, we can see that the band corresponding to the ester **16b** at $\lambda_{\text{max}} = 272 \text{ nm}$ (blue) decreases its intensity with time, appearing a new compound with a band at $\lambda_{\text{max}} 283 \text{ nm}$ (red) that corresponds to the squaramate acid analogue.

When we studied the hydrolysis reaction of **16b** at different pH , we found that neither the hydrolysis nor the cyclization of **16b** at 37°C in moderate acidic media ($\text{pH} 3\text{-}5$) progress for longer than three weeks. However, at the range of $\text{pH} 7\text{-}11$, we obtained a hydrolysis kinetic constant of $1.03 \text{ M}^{-1} \text{ s}^{-1}$

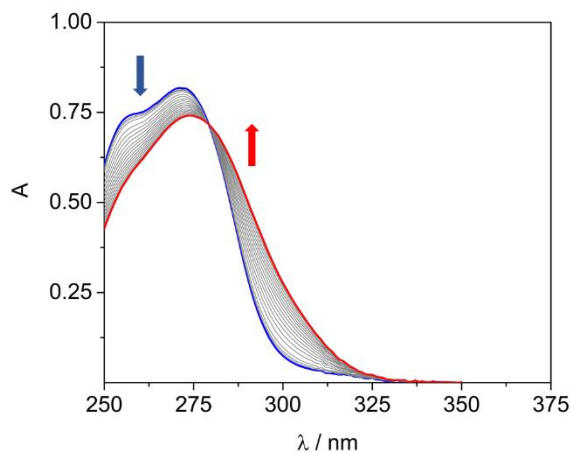
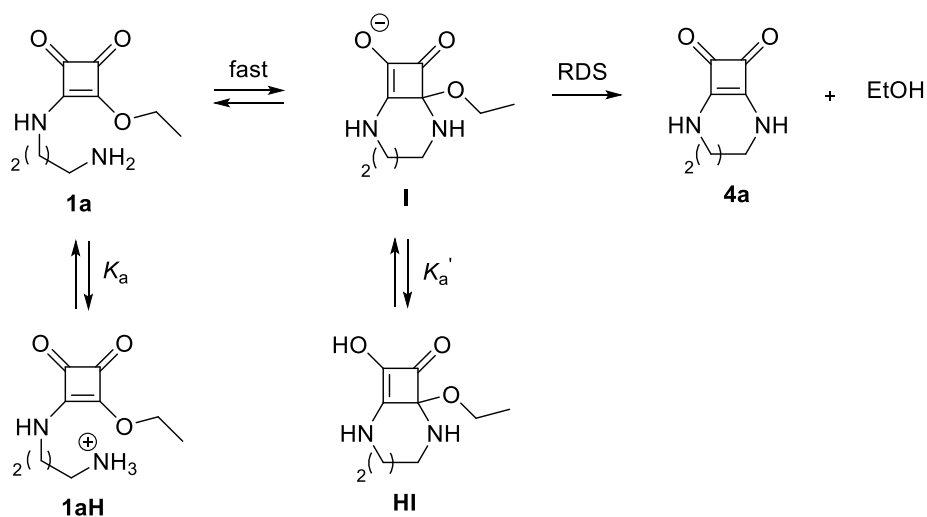


Figure S18. Changes in the UV spectra observed during the hydrolysis of 30 μM squaramate ester **16b** (30 μM , pH 7 at 37 $^{\circ}\text{C}$)

Scheme S5 Putative intramolecular addition-elimination mechanism for ester **1a** under mildly acidic conditions.⁹



Rate law of a zero order reaction (intramolecular process)

$$r = k_{\text{obs}} \quad (\text{I})$$

Rate law of a zero order reaction when $[A]$ depends on the $\text{p}K_{\text{a}}$

$$k_{\text{obs}} = k_{\text{c}}[A] = k_{\text{c}} \frac{K_{\text{a}}}{K_{\text{a}} + [\text{H}]} \quad (\text{II})$$

Rate law of a zero order reaction as a function of the concentration of an intermediate species, $[I]$

$$k_{\text{obs}} = k_{\text{c}}[I] = k_{\text{c}} \frac{K_{\text{a}}'}{K_{\text{a}}' + [\text{H}]} \quad (\text{III})$$

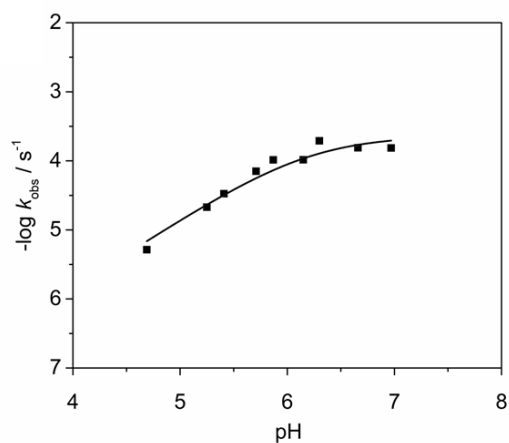


Figure S19. Plot of the k_{obs} obtained for the intermolecular cyclization reaction of ethyl squaramate **1a** at pH range 4.5-7. The curve shows the fitting of the experimental data to eq. (III)

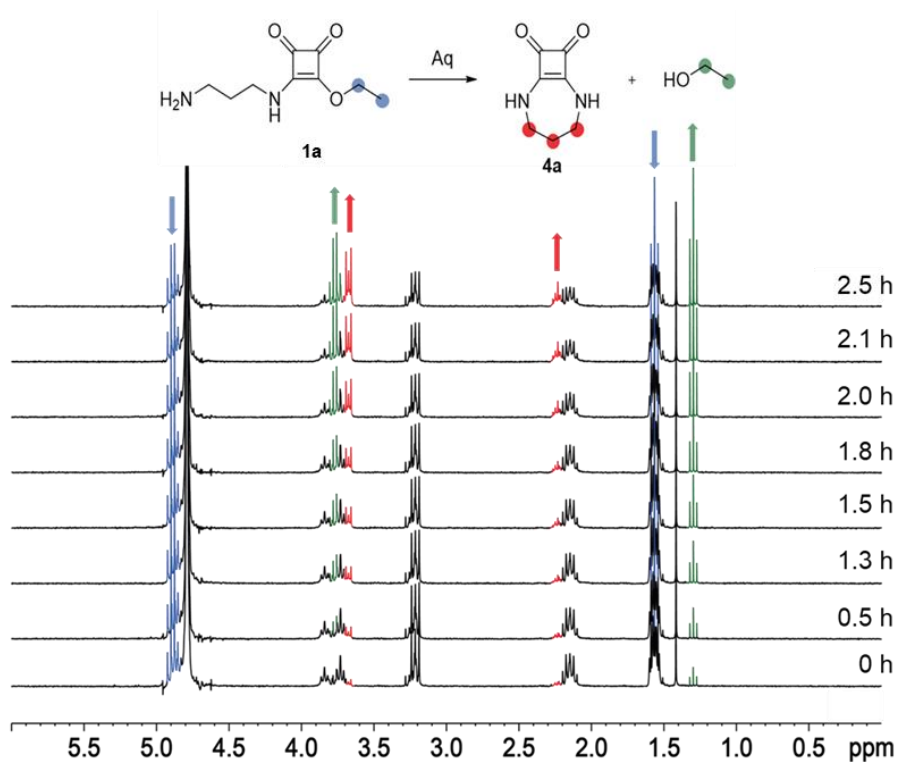


Figure S20. ¹H-NMR evolution over time of 1 mM ethyl squaramate **1a** at 0.1 M PBS at pH 7.0 (24 °C).

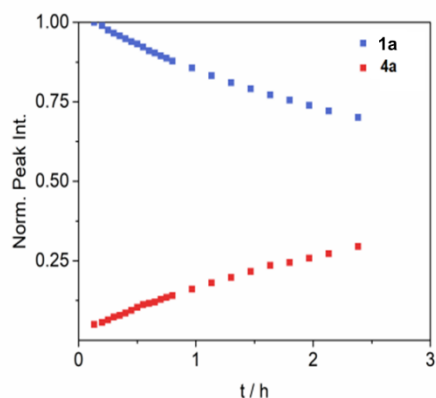


Figure S21. Representation of the calculated concentrations of **1a** and **4a** against time from eq. (V).

$$-\frac{d[\mathbf{1b}]}{dt} = \frac{d[\mathbf{4a}]}{dt} = \frac{d[\text{EtOH}]}{dt} = k_{\text{obs}}[\text{EtOH}] \quad (\text{IV})$$

$$[\text{EtOH}] = [\text{EtOH}]_0 e^{-k_{\text{obs}}t} \quad (\text{V})$$

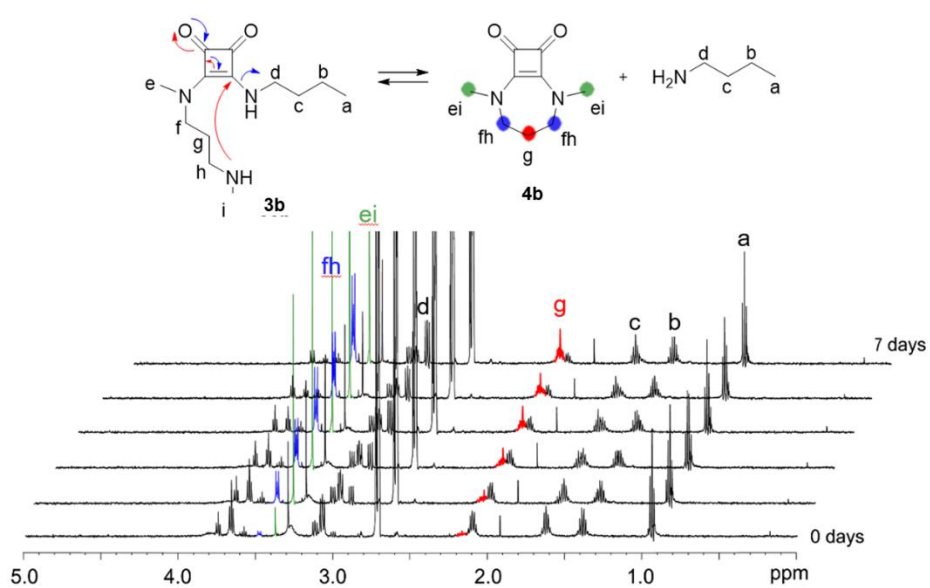


Figure S22. $^1\text{H-NMR}$ kinetic study of intramolecular cyclization of **3b** 1 mM in 0.1 M PBS pH 8, 10 % $\text{DMSO-}d_6$, 37 °C. All spectra recorded were registered using a WATERGATE pulse system for water suppression.

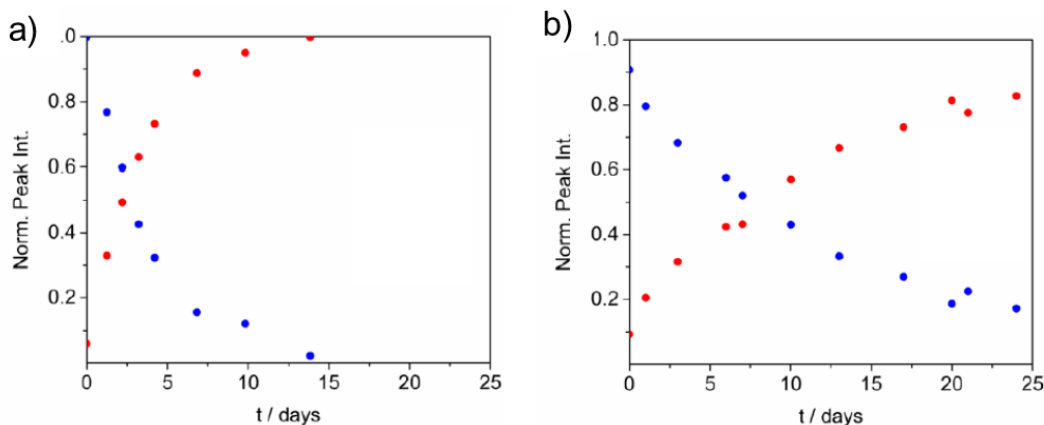


Figure S23. Variation of the concentration obtained by $^1\text{H-NMR}$ peak integration of the self-immolative compounds (a) **3b** and (b) **3c** (blue) and the cyclic squaramides **4b** and **4c** (red) upon time at pH 8 and 37 °C, respectively.

5. Cross-linking Assays

HRMS detection of DNA Cross-Linking

To determine the reactivity of compound **6** and the parent mustard **15** towards a representative DNA, we selected the Drew-Dickerson Dodecamer (**DDD**), a prototypical B-DNA with a self-complementary CGCGAATTCGCG sequence. The Drew-Dickerson Dodecamer (**DDD**) was purchased from Invitrogen (Thermo Fisher Scientific). The crude reaction products were analysed by High Resolution Mass Spectrometry (HRMS) using Electrospray Ionization (ESI) in negative mode. The initial analysis of a 10 μM solution of untreated commercial **DDD** revealed a multicharged species distribution detecting only the parent compound as a single strand DNA in three main charged states, highlighted in yellow: 1214.2109 ($z = 3$), 910.4071 ($z = 4$) and 728.1243 ($z = 5$). Figure S24.

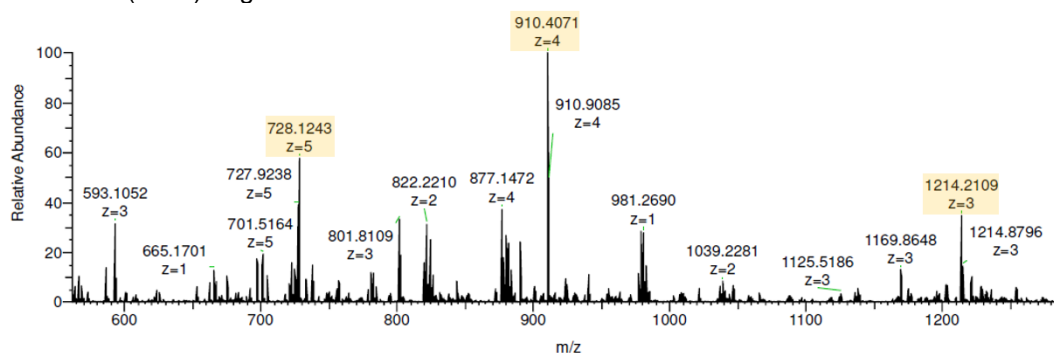


Figure S24. HRMS spectrogram, showing the multicharged species distribution of ssDDD.

Then **DDD** (10 μM) was incubated with the corresponding drug (40 μM of **15** or **6**) at 37 °C for 48 h in 1 mM Tris buffer (pH 7.4). For the activation of the self-immolative system, nitroreductase (NTR) and NADH were added (5 $\mu\text{g}/\text{mL}$ and 35 μM respectively). The solutions were injected to the mass spectrometer and ionization was performed in ESI(-) mode.

Figure S25 and S26 show the formation of single stranded mono-alkylated species of **DDD-15** and **DDD-6** in several multicharged states.

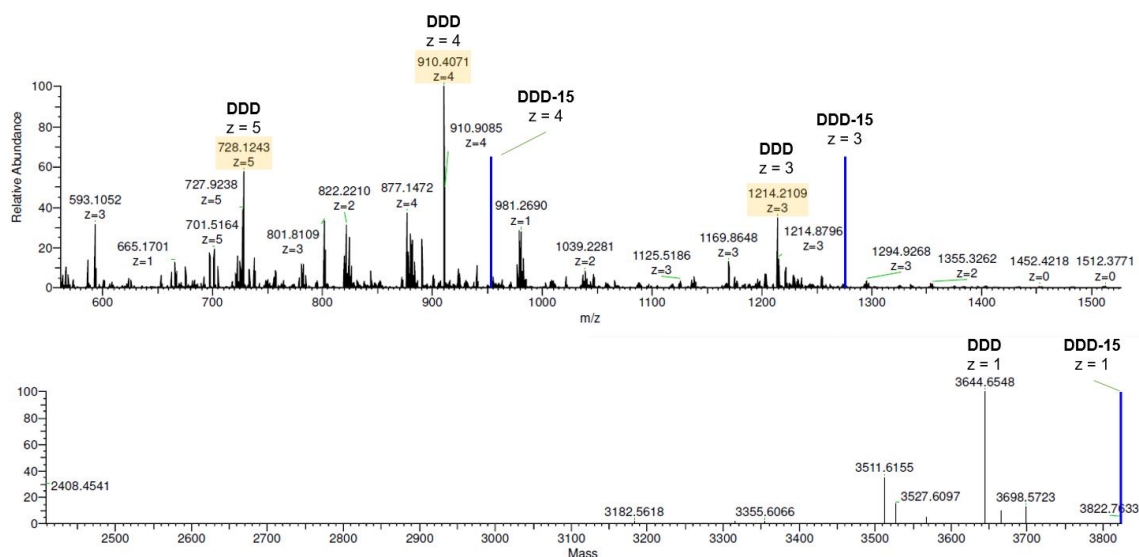


Figure S25. Spectrogram of 10 μM DDD incubated with 40 μM of **15** the alkylating Nitrogen for 48 h at 37 $^{\circ}\text{C}$

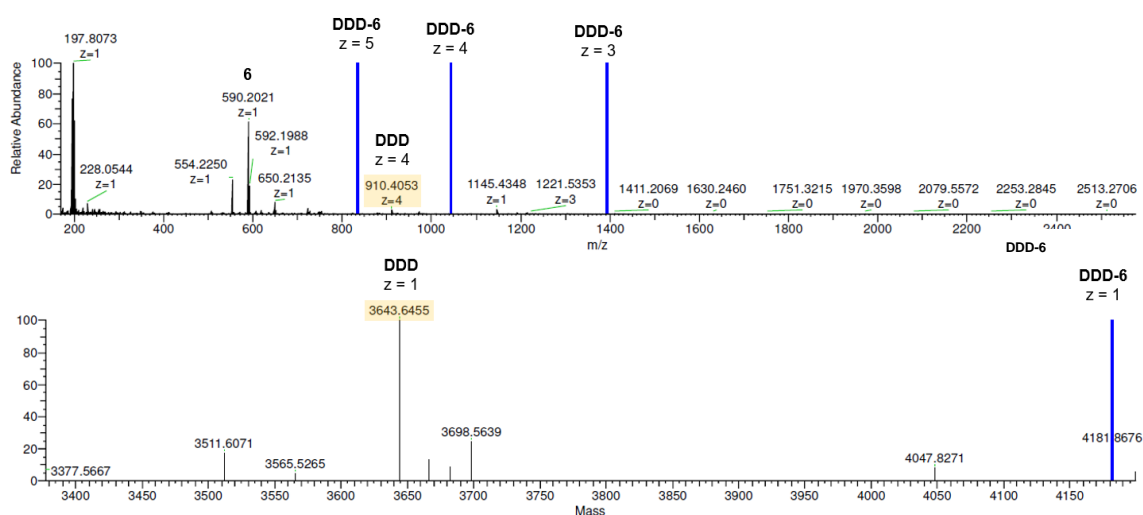


Figure S26. Spectrogram of 10 μM of ssDDD incubated with 40 μM **6** for 48 h at 37 $^{\circ}\text{C}$.

When compound **6** was incubated with DDD in presence of the NTR/NADH reduction tandem, the crude reaction analysis by HRMS ESI (-) mode showed the formation of the **DDD-15** adduct [3813.5982 ($z = 1$)], and peaks of multi charged species of the same product [$z = 3$ and $z = 4$], Figure S27. Additionally, the monoisotopic mass of the cyclic squaramide **4b** [181.0972 ($z = 1$)] was detected in ESI (+) mode (Figure S27 inset)

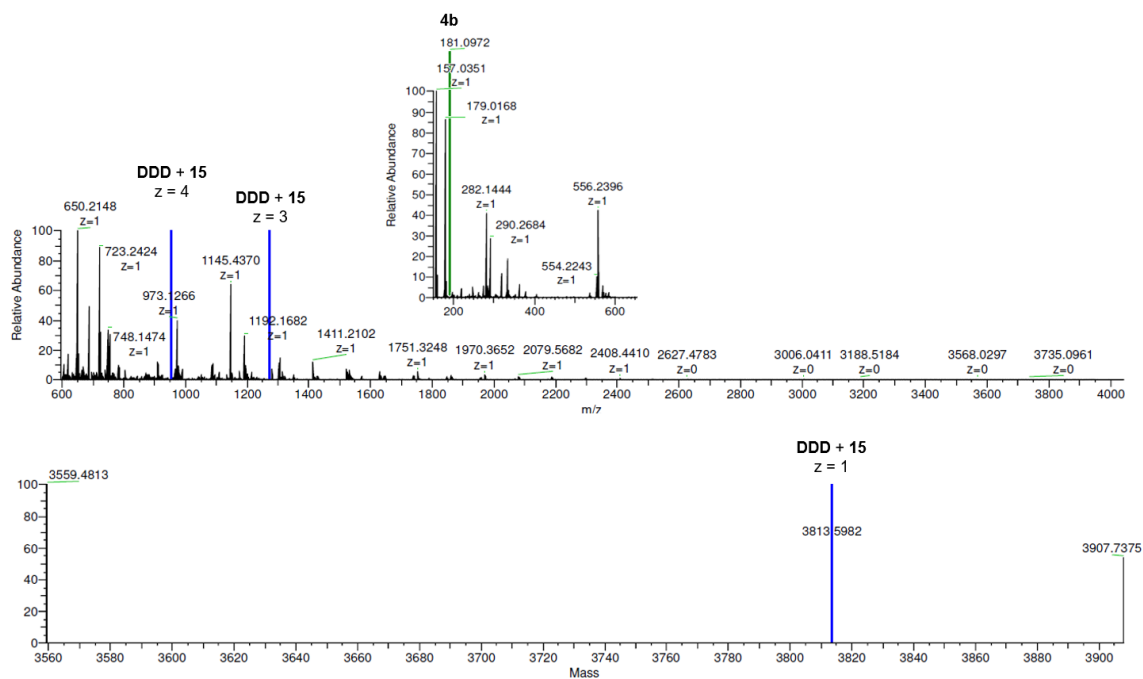


Figure S27. Spectrogram of 10 μM **ssDDD** incubated with 40 μM **6** under activation by 5 $\mu\text{g/mL}$ of NTR and 35 μM NADH for 96 h at 37 $^{\circ}\text{C}$ (ESI (-) mode). The cyclic **4b** compound was identified as well as the alkylation of the **DDD** with the **ANM** compound **15** (ESI (+) mode).

Gel retardation assay

2 μL of the stock solution of *pmax*-GFP plasmid (3486 bp, 1.0 $\mu\text{g/mL}$) were mixed with 48 μL of the alkylating agent ($2.5 \cdot 10^{-5}$ - 10^{-4} M) solution in PBS (0.1 % DMSO, 0.1 M, pH 7.4, 0.15 M NaCl). The samples were incubated at 37 $^{\circ}\text{C}$ for 48 h and subjected to 0.75 % agarose gel analysis (40 V, 90 min). The gel was incubated with Ethidium Bromide for 30 min and exposed to UV to obtain the image. For prodrug activation, 2 μL of plasmid solution were mixed with 0.75 μL of NTR solution (1 mg/mL) and/or 0.5 μL of NADH solution (50 mM), and then added up to a final volume of 50 μL with a solution of **6** (10^{-4} M).

6. Clonogenic Assay

The clonogenic survival assay was performed by modification of van Bree et al. procedure.¹⁰ 500000 cells were plated in six-well plates to a final volume of 2 mL/well. Then, the media was removed and cells were treated with the corresponding alkylating agent for 1 h. The cells were trypsinised, counted and plated at two concentrations (200 and 500 cells/well respectively). The cell culture was then allowed to grow for 9 days. At the termination of the assay, cells were rinsed with PBS. Colonies thus formed were stained for 45 minutes with 0.5 % (w/v) crystal violet prepared in 70 % (v/v) ethanol solution, rinsed with water, and finally air-dried.

Table S1. LN229 clones formed and survival factor (SF) after the treatment with antitumor agents.

Cell line / Agent	LN229 ^a		
	200 cells	500 cells	SF %
CONT	144	197	100
TMZ	83	116	58
15	155	209	106
6	30	55	24

Plate efficiency: ^a56 %

Table S2. U87-MG clones formed and survival factor (SF) after the treatment with antitumor agents.

Cell line / Agent	U87-MG ^a		
	200 cells	500 cells	SF %
CONT	127	165	100
TMZ	38	53	31
15	106	172	94
6	43	51	32

Plate efficiency: ^a48 %.

7. Cytotoxicity

Cell culture: U87-MG cells were subconfluent grown in DMEM (Life Technologies, Carlsbad, CA) supplemented with 10 % fetal calf serum (Sigma-Aldrich, St. Louis, MO) in a humidified incubator at 37 °C with 5 % CO₂. Measurement of cell viability: The number of viable cells in culture was determined based on quantification of ATP, which signals the presence of metabolically-active cells, using the Cell Titer-Glo luminiscent assay kit (Promega, Madison, WI). Following the manufacturer's instructions, 5 × 10³ cells were plated in 96-well plates, treated 24 h later with **4b** (1-200 μM), followed by addition of Cell Titer-Glo reagent after 48 h of incubation. Luminiscence was detected using a multi-well Synergy Mx scanning spectrophotometer (Biotek, Winooski, VT)

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