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Supporting Information

Tagging Live Cells that Express Specific Peptidase Activity with Solid-State Fluorescence

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Supplementary information

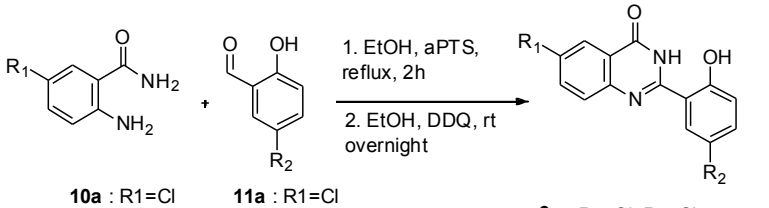
1 Chemistry

1.1 General notes

Dry dichloromethane (DCM) was obtained by passing commercially available DCM through a column containing activated alumina and under argon atmosphere. Column chromatography was performed on Merck silica gel Si-60 (40-63 µm). Routine chemicals were supplied by Sigma-Aldrich Co., Alfa Aesar, Acros organics, Tokyo Chemical Industries, Promega, and Invitrogen. They were used without further purification.

Unless stated otherwise, all spectra were acquired at 297 K on a Bruker AVANCE 300 (300 MHz & 75 MHz for ^{1}H and ^{13}C, respectively) or on a Bruker AVANCE 500 (500 MHz & 125 MHz for ^{1}H and ^{13}C, respectively) as indicated. Chemical shifts δ are reported in ppm with reference to residual solvent signals; peaks are annotated as follows: s=singlet, d=doublet, t=triplet, m=multiplet, br=broad; coupling constants *J* are given in Hertz (Hz) and refer to (H,H) coupling. Unit masses were measured by direct injection into the mass analyzer of an AGILENT 1100 SL LC-MS system running in ESI mode. HRMS data was obtained from the Centre Commun de Spectrométrie de Masse, Université Claude Bernard, Lyon, France. UV-vis spectra were recorded on a UV-670 UV-VIS spectrophotometer (JACSO Inc.) and Circular dichroism spectra were recorded on a Chirascan CD Spectrometer (Applied Photophysics).

1.2 Synthesis of the solid-state fluorophores (3a-d)



10a : R1=Cl
10b : R1=H

11a : R1=Cl
11b : R1=H

3a : R_1=Cl, R_2=Cl, quant.
3b : R_1=H, R_2=Cl, 92%
3c : R_1=Cl, R_2=H, 82%
3d : R_1=H, R_2=H, 83%

General procedure:

Compounds **3a-d** were synthesized following a similar procedure previously described by our group.[1] Briefly, anthranilamide derivative **10a** or **10b** (1 eq) was dissolved in absolute EtOH to give a red solution to which salicylaldehyde derivative **11a** or **11b** (1 eq) was added at room temperature. This reaction mixture was heated to reflux for 30 min, then p-TsOH monohydrate (0.02 eq) was added and reflux was continued for 1 h. The yellow suspension was cooled to RT, then DDQ (1.01 eq) was added in several portions and the reaction mixture was further stirred overnight at RT. The precipitate was filtered, washed twice with absolute EtOH, then twice with diethyl ether and was finally air dried to yield a beige powder showing strong green fluorescence under a UV lamp.

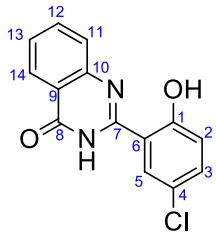
3a: 6-chloro-2-(5-chloro-2-hydroxyphenyl)quinazolin-4(3H)-one (ELF®97)

2-amino-5-chlorobenzamide **10a** (3.5 g, 20.3 mmol), 5-salicylaldehyde **11a** (3.2 g, 20.3 mmol), p-TsOH (78 mg, 0.4 mmol) and DDQ (4.7 g, 20.5 mmol) in absolute EtOH (50 mL) were used to prepare **3a** (6.24 g, 6.7 mmol) in quantitative yield.

NMR and Mass analyses match the ones previously described by J. Aw et al.[2]

3b: 2-(5-chloro-2-hydroxyphenyl)quinazolin-4(3H)-one

Anthranilamide **10b** (1.00 g, 7.3 mmol), 5-salicylaldehyde **11a** (1.14 g, 7.3 mmol), p-TsOH (29 mg, 0.15 mmol), DDQ (1.68 g, 7.4 mmol) in absolute EtOH (20 mL) were used to prepare **3b** (1.84 g, 6.7 mmol) in 92 % yield.

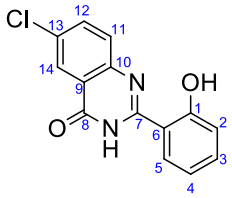


NMR: ^{1}H NMR (500 MHz, DMSO-d_6, 418 K) δ = 13.70 (br s, 1H, OH), 12.44 (br s, 1H, NH), 8.34 (d, J = 2.2 Hz, 1H, H_5), 8.16 (d, J = 7.5 Hz, 1H, H_{14}), 7.87 (t, J = 7.5 Hz, 1H, H_{12}), 7.78 (d, J = 8.1 Hz, 1H, H_{11}), 7.57 (t, J = 7.5 Hz, 1H, H_{13}), 7.48 (dd, J = 8.1, 2.2 Hz, 1H, H_3), 7.04 (d, 1H, J = 8.1Hz, H_2) ppm.

^{13}C NMR (125 MHz, DMSO-d_6, 418 K) δ = 161.1 (C_8), 158.5 (C_1), 152.6 (C_7), 145.9 (C_{10}), 134.9 (C_{12}), 133.0 (C_3), 127.1 (C_5 + C_{13}), 126.1 (C_{11}), 126.0 (C_{14}), 122.5 (C_4), 120.8 (C_9), 119.5 (C_2), 115.3 (C_6) ppm.

3c: 6-chloro-2-(2-hydroxyphenyl)quinazolin-4(3H)-one

2-amino-5-chlorobenzamide **10a** (1.00 g, 5.9 mmol), salicylaldehyde **11b** (617 µL, 5.9 mmol), p-TsOH (23 mg, 0.12 mmol), DDQ (1.36 g, 6.0 mmol) in absolute EtOH (15 mL) were used to prepare **3c** (1.32 g, 4.8 mmol) in 82 % yield.



NMR: ^{1}H NMR (500 MHz, DMSO-d_6, 418 K) δ = 13.24 (br s, 1H, OH), 12.48 (br s, 1H, NH), 8.20 (br s, 1H, H_5), 8.08 (s, 1H, H_{14}), 7.85 (s, 1H, H_{12}), 7.80 (s, 1H, H_{11}), 7.46 (s, 1H, H_3), 6.98 (m, 2H, H_2 + H_4) ppm.

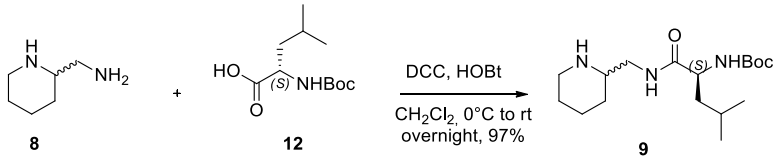
^{13}C NMR (125 MHz, DMSO-d_6, 418 K) δ = 160.2 (C_8), 159.5 (C_1), 153.8 (C_7), 145.1 (C_{10}), 134.8 (C_{12}), 133.6 (C_3), 130.9 (C_{13}), 128.3 (C_{11}), 127.9(C_5), 124.9 (C_{14}), 121.9 (C_9), 118.8(C_4), 117.7 (C_2), 113.9 (C_6) ppm.

3d: 2-(2-hydroxyphenyl)quinazolin-4(3H)-one (HPQ)

Anthranilamide **10b** (3.20 g, 23.5 mmol), salicylaldehyde **11b** (2.46 mL, 23.5 mmol), p-TsOH (86 mg, 0.45 mmol), DDQ (5.40 g, 23.8 mmol) in absolute EtOH (60 mL) were used to prepare **3d** (4.78 g, 20.1 mmol) in 83 % yield.

NMR and Mass analyses match the ones previously described by Baghbanzadeh et al.[3]

1.3 Coupling of the enzyme substrate unit with the spacer (9 and 9-(R))



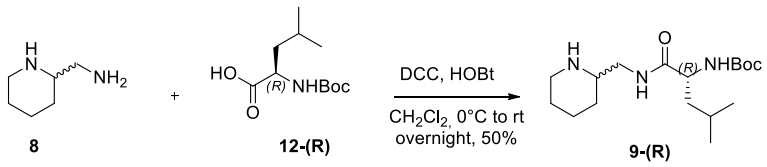
8 12 9

9: tert-butyl ((2S)-4-methyl-1-oxo-1-((piperidin-2-ylmethyl)amino)pentan-2-yl)carbamate

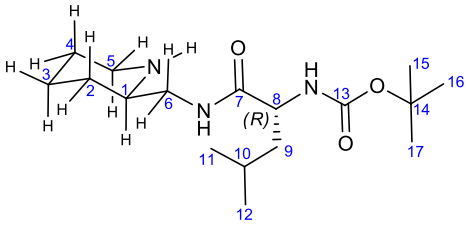
To an ice-cold suspension of Boc-protected L-leucine **12** (8.0 g, 28.9 mmol) and HOBt (4.8 g, 32.0 mmol, 1.1 eq) in DCM (60 mL) was added dropwise a solution of dicyclohexylcarbodiimid (7.3 g, 32.0 mmol, 1.1 eq) in DCM (25 mL). The resulting mixture was stirred at 0°C for 1 h and a further 30 min at RT. Then 2-(aminomethyl)piperidine **8** (3.99 mL, 28.4 mmol, 1.0 eq) was added dropwise to

the milky reaction mixture and the resulting suspension was stirred at RT overnight. The solid material (dicylohexylurea) was filtered off and the filtrate was washed with a saturated solution of $NHCO_3$ (2x100 mL). The organic layer was extracted several times with a 10 wt-% aqueous solution of KH_2PO_4 at pH≈3 until the pH of the aqueous layer remains stable at this value. DCM (150 mL) was added to the combined acidic aqueous layers which are then treated with an aqueous solution of 2M NaOH to reach a pH of 12. This basic aqueous layer is washed twice with DCM (2x100 mL) and the combined organic layers are dried with anhydrous Na_2SO_4 and evaporated under reduced pressure to give **9** as a white solid (9.2 g, yield: 97 %).

NMR and mass analysis matches the ones previously described by our group.[1]



9-(R) was synthesized following a similar procedure starting from Boc-D-Leu-OH 12-(R) (1.0 g, 4.24 mmol), HOBt (630 mg, 4.66 mmol, 1.1 eq), DCC (971 mg, 4.66 mmol, 1.1 eq) and 2-(aminomethyl)piperidine 8 (530 µL, 4.24 mmol, 1.0 eq) in DCM (15 mL) to obtain **9-(R)** as a white powder (700 mg, 2.12 mmol, yield: 50 %)

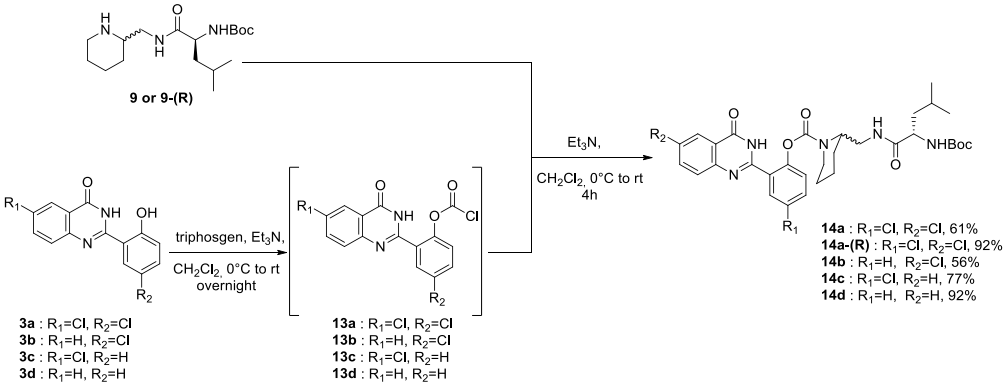


NMR: ^{1}H-NMR (300 MHz, $CDCl_3$): δ= 6.58 (m, 1H, NH amide), 5.08 (m, 1H, $NH_{carbamate}$), 4.05 (m, 1H, H_8), 3.33-3.20 (m, 1H, H_{6a}), 3.13-3.13 (m, 2H, H_{5a} + H_{6b}), 2.67-2.54 (m, 2H, H_1 + H_{5b}), 1.78-1.75 (m, 1H, H_{4a}), 1.66-1.46 (m, 6H, NH_{amine} + H_{2a} + H_{3a} + 2xH_9 + H_{10}), 1.42 (s, 9H, 3×H_{15} + 3×H_{16} + 3×H_{17}), 1.39-1.30 (m, 2H, H_{2b} + H_{4b}), 1.14-1.01 (m, 1H; H_{3b}), 0.93-0.90 (2d, 6H, ^{3}J=6.06Hz 3xH_{11} + 3xH_{12}) ppm.

^{13}C-NMR (125 MHz, $CDCl_3$): δ= 156.3 (C_7), 79.3 (C_8), 56.3 (C_1), 46.9 (C_6), 46.7 (C_5), 30.4 (C_3), 28.5 (C_9 + C_{10} + C_{11}), 26.6 (C_2), 24.4 (C_4) ppm.

MS: ESI: $[M+H]^+$ m/z found 328.3, calc. 328.3

1.4 Coupling of spacer-substrate unit to solid-state fluorophores (14a-d)

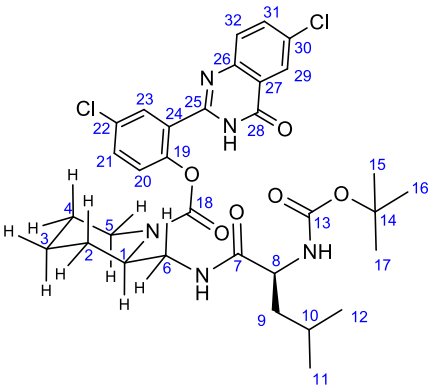


General procedure:

To an ice-cold suspension of the HPQ derivative **3a-d** in dry DCM under an argon atmosphere was added dropwise freshly distilled triethylamine stored on KOH (2 eq) followed by a solution of triphosgene (3.5 eq) in dry DCM. This solution was stirred at 0°C for 1 h and overnight at RT. The resulting mixture is then evaporated to dryness under reduced pressure and the volatiles are trapped in a liquid nitrogen trap. **--CAUTION**: the volatiles contain highly toxic phosgene which has to be quenched with a mixture of EtOH in DCM (1:1 v:v) before disposal-- The resulting solid residue containing **13a-d** was resuspended in dry DCM and cooled to 0°C before a solution of **9** (0.5 eq) in dry DCM was added dropwise followed by triethylamine (4.0 eq). The progression of the reaction was followed by TLC (petroleum ether / ethyl acetate, 6:4 v:v). For the ease of purification, a solution of piperidine in pyridine can be added at the end of the reaction to trap the rest of the HPQ chloroformate avoiding the release of free HPQ which is difficult to get rid of. The reaction mixture was then diluted with DCM and washed twice with sat. $NaHCO_3$ and once with brine. The organic layer was dried over Na_2SO_4 and the volatiles were removed under reduced pressure to give the crude product. This mixture was purified via column chromatography on silicagel (petroleum ether / ethyl acetate, 8:2, 7:3, 6:4, 5:5 v/v) to obtain **14a-d**.

14a: (S)-4-chloro-2-(6-chloro-4-oxo-3,4-dihydroquinazolin-2-yl)phenyl 2-((2-((tert-butoxycarbonyl)-amino)-4-methylpentanamido)methyl)piperidine-1-carboxylate

3a (1.27 g, 3.67 mmol), triphosgene (3.30 g, 11.0 mmol), triethylamine (1.05 mL, 7.34 mmol), in dry DCM (30 + 20 mL) were used to produce intermediate **13a**. **9** (600 mg, 1.83 mmol), triethylamine (2.1 mL, 14.7 mmol) in dry DCM (30 mL) were used to produce **14a** (720 mg, 1.09 mmol) in 61 % yield.



NMR: ^{1}H-NMR ($CDCl_3$, 500 MHz) δ = 11.45+11.39+11.21+10.92 (m, 1H, $NH_{fluorophore}$), 8.38 + 8.23 + 8.19 + 8.01 + 7.94 + 7.82 + 7.73 + 7.63+ 7.40 + 7.21 + 7.07 + 6.84 (m, 7H, NH_{amide} + 6x$H_{aromatic}$), 5.27 + 5.11 + 4.97 (m, 1H, $NH_{carbamate}$), 4.61 + 4.30 + 4.06 (m, 2H, H_1 + H_8), 4.06-2.87 (m, 4H, 2×H_5+2×H_6), 1.72–1.40 (m, 9H, 2xH_2 + 2×H_4 + 2xH_3 + 2xH_9 + H_{10}), 1.34 + 1.23 + 1.06 + 0.98 (4xs; 9H, 3×H_{15} + 3×H_{16} + 3×H_{17}), 0.94-0.86 (m, 6H, 3×H_{11} + 3xH_{12}) ppm.

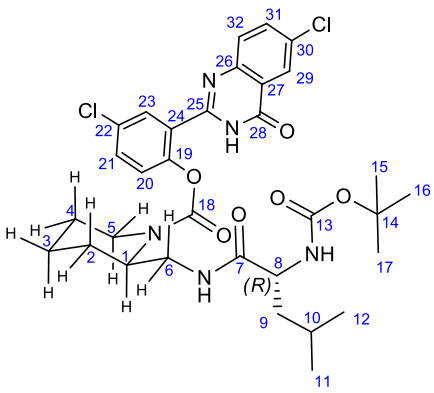
^{13}C-NMR ($CDCl_3$, 125 MHz): δ = 174.6 + 174.0 + 173.9 + 173.4 (C_7), 162.2 + 162.0 + 161.9 + 161.2 (C_{28}), 156.1 + 155.9 + 155.7 + 154.0 + 152.7 + 152.3 + 151.7 + 150.4 + 150.3 + 150.1 + 149.7 + 148.3 + 148.1 + 148.1 + 148.0 + 147.7 + 147.5 + 147.4 (C_{13} + C_{18} + C_{25} + C_{19} + C_{26}), 135.3 + 135.2 + 133.2 + 133.0 + 132.8 + 132.1 + 131.7 + 131.6 + 131.4 + 130.5 + 130.4 + 130.2 + 129.9 + 129.6 + 129.3 + 129.1 + 129.0 + 128.1 + 125.9 + 125.6 + 124.9 + 124.4 + 123.9 + 122.1 + 122.0 + 121.8 (C_{31} + C_{30} + C_{21} + C_{23} + C_{22} + C_{32} + C_{27} + C_{29} + C_{20} + C_{24}), 80.2 + 79.8 (C_{14}), 54.3 + 53.2 + 53.0 + 52.6 + 52.3 + 52.0 + 50.6 (C_1 + C_8), 41.6 + 41.4 + 41.3 + 41.0 (C_9), 40.4 + 39.8 + 39.7 + 39.0 + 38.7 (C_5 + C_6), 28.2 +27.9 + 27.8 (C_{15} + C_{16} + C_{17}), 26.8 + 26.5, 25.5 + 25.3 + 25.2 (C_2 + C_4), 24.8 + 24.8 (C_{10}), 23.1 + 22.8 + 22.2 + 21.7 (C_{11} + C_{12}), 19.4 + 19.0 + 18.9 (C_3) ppm.

R_f = 0.35 (petroleum ether / ethyl acetate 6 : 4 v/ v) – revealed by UV and ninhydrine

HRMS: $C_{32}H_{40}ClN_5O_4$ $[M+H]^+$ m/z found: 660.2342 calc. 660.2350

14a-(R): (S)-4-chloro-2-(6-chloro-4-oxo-3,4-dihydroquinazolin-2-yl)phenyl-2-((2-((tertbutoxycarbonyl)-amino)-4-methylpentanamido)methyl)piperidine-1-carboxylate

3a (308 mg, 1.00 mmol, 1.1 eq), triphosgene (900 mg, 3.00 mmol, 3.3 eq), diisopropylethylamide (520 µL, 3.00 mmol, 3.3 eq), in dry DCM (10 + 4 mL) were used to produce intermediate **13a**. **9-(R)** (300 mg, 0.91 mmol, 1.0 eq), diisopropylethylamide (630 µL, 3.65 mmol, 4.0 eq) in dry DCM (10 mL) were used to produce **14a-(R)** (562 mg, 0.85 mmol) in 92 % yield.



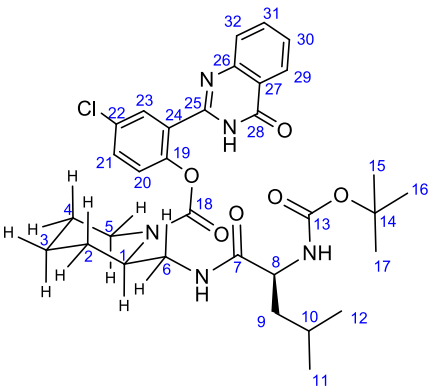
NMR: ^{1}H-NMR ($CDCl_3$, 500 MHz) δ = 11.41+11.38+11.13+10.62 (m, 1H, $NH_{fluorophore}$), 8.43 + 8.27 + 8.21 + 8.05 + 8.0 + 7.92 + 7.85 + 7.73 + 7.64+ 7.43 + 7.23 + 7.06 + 6.71 (m, 7H, NH_{amide} + 6x$H_{aromatic}$), 5.27 + 4.98 + 4 .86 (m, 1H, $NH_{carbamate}$), 4.64 + 4.30 + 4.10 (m, 2H, H_1 + H_8), 4.07-2.84 (m, 4H, 2×H_5+2×H_6), 1.72–1.40 (m, 9H, 2xH_2 + 2×H_4 + 2xH_3 + 2xH_9 + H_{10}), 1.34 + 1.23 + 1.06 + 0.98 (4xs; 9H, 3×H_{15} + 3×H_{16} + 3×H_{17}), 0.94-0.86 (m, 6H, 3×H_{11} + 3xH_{12}) ppm.

^{13}C-NMR ($CDCl_3$, 125 MHz): δ = 174.5 + 174.0 + 173.9 + 173.4 (C_7), 162.2 + 162.0 + 161.9 + 161.2 (C_{28}), 156.1 + 155.9 + 155.7 + 154.0 + 152.7 + 152.3 + 151.7 + 150.4 + 150.3 + 150.1 + 149.7 + 148.3 + 148.1 + 148.1 + 148.0 + 147.7 + 147.5 + 147.4 (C_{13} + C_{18} + C_{25} + C_{19} + C_{26}), 135.3 + 135.2 + 133.2 + 133.0 + 132.8 + 132.1 + 131.7 + 131.6 + 131.4 + 130.5 + 130.4 + 130.2 + 129.9 + 129.6 + 129.3 + 129.1 + 129.0 + 128.1 + 125.9 + 125.6 + 124.9 + 124.4 + 123.9 + 122.1 + 122.0 + 121.8 (C_{31} + C_{30} + C_{21} + C_{23} + C_{22} + C_{32} + C_{27} + C_{29} + C_{20} + C_{24}), 80.2 + 79.8 (C_{14}), 54.3 + 53.2 + 53.0 + 52.6 + 52.3 + 52.0 + 50.6 (C_1 + C_8), 41.6 + 41.4 + 41.3 + 41.0 (C_9), 40.4 + 39.8 + 39.7 + 39.0 + 38.7 (C_5 + C_6), 28.2 +27.9 + 27.8 (C_{15} + C_{16} + C_{17}), 26.8 + 26.5, 25.5 + 25.3 + 25.2 (C_2 + C_4), 24.8 + 24.8 (C_{10}), 23.1 + 22.8 + 22.2 + 21.7 (C_{11} + C_{12}), 19.4 + 19.0 + 18.9 (C_3) ppm.

R_f = 0.35 (petroleum ether / ethyl acetate 6 : 4 v/v) – revealed by UV and ninhydrine

14b: (S)-4-chloro-2-(4-oxo-3,4-dihydroquinazolin-2-yl)phenyl 2-((2-((tert-butoxycarbonyl)amino)-4-methylpentanamido)methyl)piperidine-1-carboxylate

3b (500 mg, 1.83 mmol), triphosgene (1.95 g, 6.5 mmol), triethylamine (550 µL, 3.8 mmol), in dry DCM (15 + 10 mL) were used to produce intermediate **13b**. **9** (300 mg, 0.92 mmol), triethylamine (1.05 mL, 7.6 mmol) in dry DCM (15 mL) were used to produce **14b** (320 mg, 0.51 mmol) in 56 % yield.



NMR: ^{1}H-NMR ($CDCl_3$, 500 MHz) δ = 11.36-10.75 (m, 1H, $NH_{fluorophore}$), 8.56 + 8.32 + 8.27 + 8.08 + 8.01 + 7.94 + 7.83 + 7.70 + 7.52 + 7.44 + 7.23 + 7.09 + 6.91 (m, 8H, NH_{amide} + 7x$H_{aromtic}$), 5.38 + 5.12 + 4.98 (m, 1H, $NH_{carbamate}$), 4.64 + 4.32 + 4.10 (m, 2H, H_1 + H_8), 4.08 + 3.92 + + 3.75 + 3.57 + 3.37 + 3.20 + 2.90 (m, 4H, 2xH_5 + 2xH_6), 1.89 + 1.63-1.57 + 1.45 + 1.36 (m, 9H, 2x H_2 + 2xH_3 + 2×H_4 + 2xH_9 + H_{10}), 1.36 + 1.20 + 1.09 + 1.02

(4xs; 9H, 3×H_{15} + 3×H_{16} + 3×H_{17}), 1.00-0.84 (m, 6H, 3×H_{11} + 3xH_{12}) ppm.

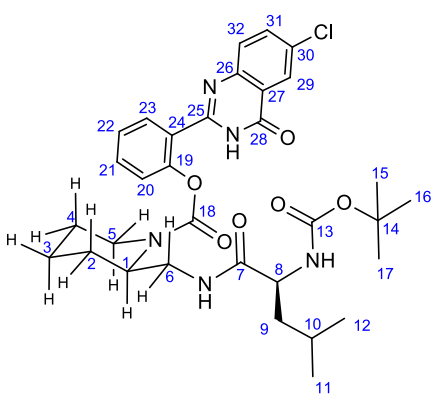
^{13}C-NMR ($CDCl_3$, 125 MHz): δ = 174.7 + 174.2 + 174.0 + 173.5 (C_7), 163.3 + 163.1 + 163.0 + 162.3 (C_{28}), 156.2 + 156.0 + 155.7 + 154.3 + 152.9 + + 152.5 + 151.8 + 150.3 + 150.1 + 150.0 + 149.7 + 149.5 + 149.3 + 149.2 + 149.0 + 148.5 + 148.3 + 148.1 + 147.8 (C_{13} + C_{18} + C_{19} + C_{25} + C_{26}), 135.1 + 134.9 + 132.0 + 131.7 + 131.6 + 131.5 + 130.6 + 130.4 + 130.2 + 129.5 + 129.4 + 128.5 + 128.4 + 128.0 + 127.5 + 127.4 + 127.1 + 126.7 + 126.4 + 126.2 + 126.1 + 125.0 + 124.5 + 124.0 + 121.2 + 121.1 + 121.0 + 120.9 (C_{31} + C_{30} + C_{21} + C_{23} + C_{22} + C_{32} + C_{27} + C_{29} + C_{20} + C_{24}), 80.2 + 79.8 (C_{14}), 54.3 + 53.2 + 53.1 + 52.6 + 52.4 + 52.1 + 51.4 + 50.6 (C_1 + C_8), 41.6 + 41.4 + 41.3 (C_9), 41.1 + 41.0 + 40.4 + 39.8 + 39.7 + 39.0 + 38.9 + 38.7 (C_5 + C_6), 28.3 +28.0 + 27.9 (C_{15} + C_{16} + C_{17}), 29.8 + 29.3 + 27.0 + 26.7 + 25.6 + 25.4 + 25.3 (C_2 + C_4), 24.9 (C_{10}), 23.2 + 23.0 + 22.9 + 22.8 + 22.4 + 22.3 (C_{11} + C_{12}), 19.6 + 19.1 + 19.0 (C_3) ppm.

HRMS: $C_{32}H_{40}ClN_5O_4$ $[M+H]^+$ m/z found: 626.2717 calc. 626.2740

R_f = 0.44 (cyclohexane / ethyl acetate 5 : 5 v/v) – revealed by UV and ninhydrine

14c: (S)-2-(6-chloro-4-oxo-3,4-dihydroquinazolin-2-yl)phenyl 2-((2-((tert-butoxycarbonyl)amino)-4-methylpentanamido)methyl)piperidine-1-carboxylate

3c (1.00 g, 3.67 mmol), triphosgene (3.90 g, 18.3 mmol), triethylamine (1.07 mL, 7.33 mmol), in dry DCM (15 + 10 mL) were used to produce intermediate **13c**. **9** (600 mg, 1.83 mmol), triethylamine (2.1 mL, 14.7 mmol) in dry DCM (15 mL) were used to produce **14c** (883 mg, 1.41 mmol) in 77 % yield.



NMR: ^{1}H-NMR ($CDCl_3$, 500 MHz) δ = 11.61-10.65 (m, 1H, $NH_{fluorophore}$), 8.41 + 8.26 + 8.20 + 8.05 + 7.98 + 7.86 + 7.71 + 7.62 + 7.51 + 7.33 + 7.27 + 7.12 + 6.79 (m, 8H, NH_{amide} + 7x$H_{aromtic}$), 5.48 + 5.07 + 4.91 (m, 1H, $NH_{carbamate}$), 4.63 + 4.34 + 4.25 + 4.08 (m, 2H, H_1 + H_8), 4.08 + 3.50 + 3.35 + 3.22 + 2.88 (m, 4H, 2xH_5 + 2xH_6), 1.86 + 1.63 + 1.57 + 1.45 + 1.34 (m, 9H, 2x H_2 + 2xH_3 + 2×H_4 + 2xH_9 + H_{10}), 1.34 + 1.20 + 1.05 + 0.96 (4xs; 9H, 3×H_{15} + 3×H_{16} + 3×H_{17}), 1.00-0.84 (m, 6H, 3×H_{11} + 3xH_{12}) ppm.

^{13}C-NMR ($CDCl_3$, 125 MHz): δ = 174.7 + 174.0 + 173.9 + 173.5 (C_7), 162.4 + 162.0 (C_{28}), 156.2 + 156.0 + 155.7 + 153.37 + 152.8 + 152.1 + 151.8 + 151.6 + 151.5 + 150.9 + 149.7 + 149.5 + 149+32 + 148.4 + 148.0 + 147.9 + 147.8 (C_{13} + C_{18} + C_{19} + C_{25} + C_{26}), 135.3 + 135.1 + 133.0 + 132.8 + 132.5 + 132.1 + 132.0 + 131.0 + 130.6 + 130.0 + 129.8 + 129.6 + 129.0 + 127.8 + 127.2 + 126.5 + 126.2 + 126.0 + 125.7 125.1 + 124.9 + 124.5 + 123.1 + 122.6 + 122.2 + 122.1 + 122.0 + 121.9 (C_{31} + C_{30} + C_{21} + C_{23} + C_{22} + C_{32} + C_{27} + C_{29} + C_{20} + C_{24}), 80.2 + 79.8 (C_{14}), 54.4 + 53.5 + 53.2 + 52.6 + 52.3 + 52.1 + 50.8(C_1 + C_8), 41.6 + 41.3 + 41.1 (C_9), 41.0 + 40.4 + 39.9 + 39.8 + 39.7 + 39.2 + 39.0 + 38.0 (C_5 + C_6), 28.3 +28.2 + 28.0 (C_{15} + C_{16} + C_{17}), 29.8 + 29.4 + 26.9 + 26.8 + 25.6 + 25.5 + 25.3 (C_2 + C_4), 24.9 (C_{10}), 23.2 + 22.9 + 22.3 + 21.7 (C_{11} + C_{12}), 19.6 + 19.2 + 19.0 (C_3) ppm.

HRMS: $C_{32}H_{40}ClN_5O_4$ $[M+H]^+$ m/z found: 626.2715 calc. 626.2740

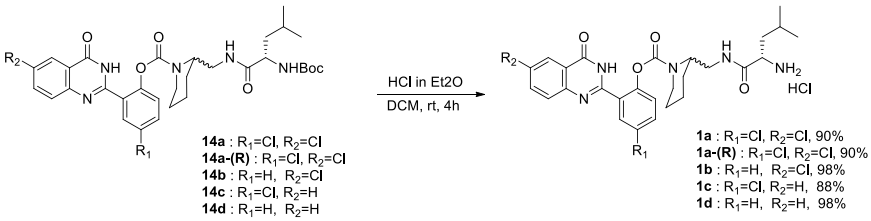
R_f = 0.28 (cyclohexane / ethyl acetate 6 : 4 v/v) – revealed by UV and ninhydrine

14d: (S)-2-(4-oxo-3,4-dihydroquinazolin-2-yl)phenyl 2-((2-((tert-butoxycarbonyl)amino)-4-methylpentanamido)methyl)piperidine-1-carboxylate

3d (1.00 g, 4.20 mmol), triphosgene (4.50 g, 14.7 mmol), triethylamine (1.2 mL, 8.40 mmol), in dry DCM (15 + 10 mL) were used to produce intermediate **13d**. **9** (690 mg, 2.10 mmol), triethylamine (2.4 mL, 16.8 mmol) in dry DCM (10 mL) were used to produce **14d** (1.150 g, 1.94 mmol) in 92 % yield.

NMR and mass analysis matches the ones previously described by our group.[1]

1.5 Deprotection toward final fluorogenic probes (1a-d)

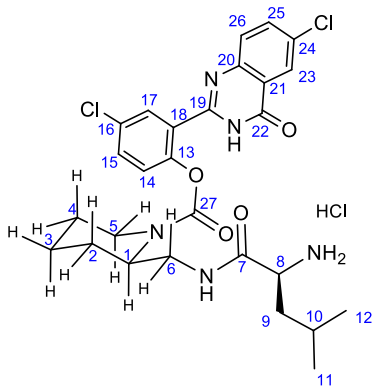


General procedure:

To a solution of protected compound **14a-d** in dry DCM under an argon atmosphere was added a solution of 2M HCl in Et_2O at RT and the reaction mixture is stirred at this temperature until the reaction TLC was complete (TLC monitoring: petroleum ether / ethyl acetate 6:4 v/v). Addition of further portions of HCl in Et_2O may be required. A white precipitate quickly appears in the mixture. At the end of the reaction this precipitate is filtered off, washed twice with a mixture of DCM / Et_2O 1:1 v/v, and twice with pure Et_2O. The solid was collected and traces of solvent evaporated overnight under reduced pressure to obtain **1a-d** as a white powder.

1a: (S)-4-chloro-2-(6-chloro-4-oxo-3,4-dihydroquinazolin-2-yl)phenyl 2-((2-amino-4-methylpentanamido)methyl)piperidine-1-carboxylate hydrochloride

14a (200 mg, 0.3 mmol), 2M HCl in Et_2O (3 mL, 6.0 mmol), in dry DCM (3 mL) were used to produce **1a** (160 mg, 0.27 mmol) in 90 % yield.



NMR: ^{1}H-NMR (CD_3OD, 500 MHz) δ = 8.24 (s, 1H, H_{23}), 7.92 (d, 1H J = 8.3Hz, H_{25}), 7.88 (s, 1H, H_{17}), 7.79 (d, 1H, J = 8.3Hz, H_{26}), 7.70 (d, 1H, J = 8.3Hz, H_{15}), 7.53 + 7.48 + 7.40 (d + d + t, 1H, J = 8.3Hz, H_{14}), 4.57 + 4.20 (br s, 1H, H_1), 4.24 + 3.87 + 3.74 + 3.48 + 3.25 + 3.16 + 2.95 (m, 4H, 2xH_5 + 2xH_6), 3.87 + 3.74 (m, 1H overlapped, H_8), 1.67-1.30 (m, 9H, 2xH_2 + 2xH_3 + 2×H_4 + 2xH_9 + H_{10}), 0.96 + 0.90 (2xs, 6H, 3×H_{11} + 3xH_{12}) ppm.

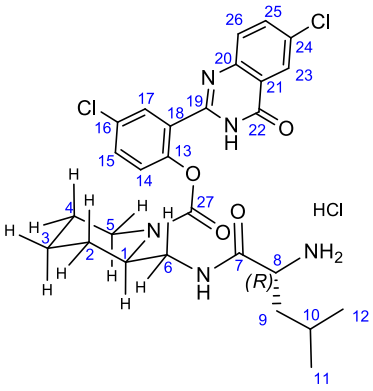
^{13}C-NMR (CD_3OD, 125 MHz): δ = 170.7 (C_7), 162.0 (C_{22}), 154.4 + 154.1 + 154.0 + 153.9 + 153.8 (C_{19} + C_{27}), 149.2 + 149.1 (C_{13}), 145.3 + 145.2 (C_{20}) 136.9 (C_{25}), 134.0 + 133.9 (C_{15}), 131.1 (C_{17}), 128.2 + 128.0 + 127.9 (C_{26}), 127.0 (C_{23}), 126.3 (C_{14}), 135.0 + 134.9 + 123.1 + 132.4 + 123.0 (C_{16} + C_{18} + C_{21} + C_{24}), 53.0 (C_8), 52.8 + 52.7 + 52.4 + 52.3 (C_1), 41.8 + 41.7 + 41.6 + 41.5 + 41.2 + 39.8 + 39.4 + 39.3 (C_5 + C_6 + C_9), 27.1 + 26.9 + 26.4 + 26.0 (C_2 + C_4), 25.4 (C_{10}), 23.2 + 21.9 (C_{11} + C_{12}), 19.8 + 19.7 + 19.5 (C_3) ppm.

HRMS: $C_{27}H_{32}Cl_2N_5O_4$ $[M+H]^+$ m/z found: 560.1808 calc. 560.1826

R_f = 0.61 (DCM / MeOH 9 : 1 v/v) – revealed by UV and ninhydrine

1a-(R): (S)-4-chloro-2-(6-chloro-4-oxo-3,4-dihydroquinazolin-2-yl)phenyl 2-((2-amino-4-methylpentan-amido)methyl)piperidine-1-carboxylate hydrochloride

14a-(R) (200 mg, 0.3 mmol), 2M HCl in Et_2O (3 mL, 6.0 mmol), in dry DCM (3 mL) were used to produce **1a-(R)** (160 mg, 0.27 mmol) in 90 % yield.

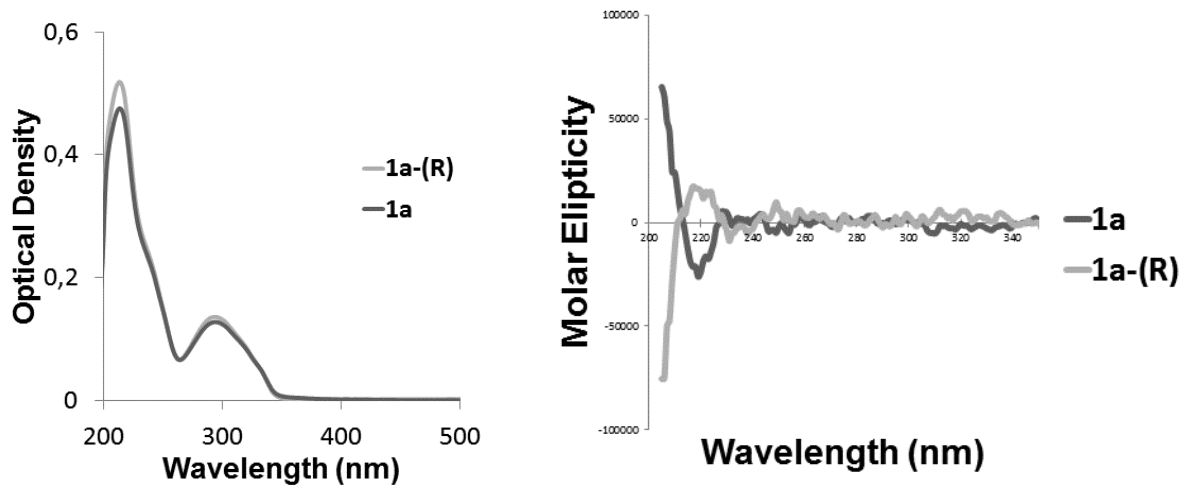


NMR: ^{1}H-NMR (CD_3OD, 500 MHz) δ = 8.24 (s, 1H, H_{23}), 7.92 (d, 1H J = 8.3Hz, H_{25}), 7.88 (s, 1H, H_{17}), 7.78 (d, 1H, J = 8.3Hz, H_{26}), 7.70 (d, 1H, J = 8.3Hz, H_{15}), 7.51 + 7.47 + 7.39 (d + d + t, 1H, J = 8.3Hz, H_{14}), 4.57 + 4.20 (br s, 1H, H_1), 4.24 + 3.87 + 3.74 + 3.48 + 3.25 + 3.16 + 2.95 (m, 4H, 2xH_5 + 2xH_6), 3.87 + 3.74 (m, 1H overlapped, H_8), 1.67-1.30 (m, 9H, 2xH_2 + 2xH_3 + 2×H_4 + 2xH_9 + H_{10}), 0.96 + 0.90 (2xs, 6H, 3×H_{11} + 3xH_{12}) ppm.

^{13}C-NMR (CD_3OD, 125 MHz): δ = 170.7 (C_7), 162.0 (C_{22}), 154.4 + 154.1 + 154.0 + 153.9 + 153.8 (C_{19} + C_{27}), 149.2 + 149.1 (C_{13}), 145.3 + 145.2 (C_{20}) 136.9 (C_{25}), 134.0 + 133.9 (C_{15}), 131.1 (C_{17}), 128.2 + 128.0 + 127.9 (C_{26}), 127.0 (C_{23}), 126.3 (C_{14}), 135.0 + 134.9 + 123.1 + 132.4 + 123.0 (C_{16} + C_{18} + C_{21} + C_{24}), 53.0 (C_8), 52.8 + 52.7 + 52.4 + 52.3 (C_1), 41.8 + 41.7 + 41.6 + 41.5 + 41.2 + 39.8 + 39.4 + 39.3 (C_5 + C_6 + C_9), 27.1 + 26.9 + 26.4 + 26.0 (C_2 + C_4), 25.4 (C_{10}), 23.2 + 21.9 (C_{11} + C_{12}), 19.8 + 19.7 + 19.5 (C_3) ppm.

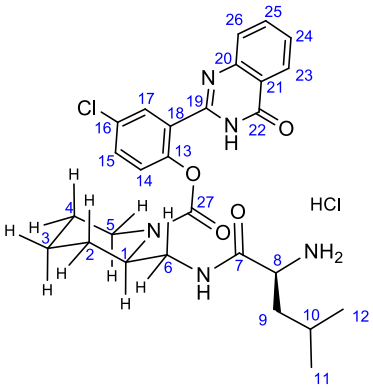
R_f = 0.61 (DCM / MeOH 9 : 1 v/v) – revealed by UV and ninhydrine

UV-vis and circular dichroism spectra of 1a and 1a-(R) (1.10^{-5} M in MeOH in a 1 cm cuvette):



1b: (S)-4-chloro-2-(4-oxo-3,4-dihydroquinazolin-2-yl)phenyl 2-((2-amino-4-methylpentanamido)-methyl)piperidine-1-carboxylate hydrochloride

14b (220 mg, 0.35 mmol), 2M HCl in Et_2O (3 mL, 6.0 mmol), in dry DCM (3 mL) were used to produce **1b** (193 mg, 0.34 mmol) in 98 % yield.



NMR: ^{1}H-NMR (D_2O, 500 MHz) δ = 8.21 (d, 1H, J = 7.7Hz, H_{23}), 7.95 (t, 1H, J = 7.7Hz, H_{25}), 7.78 (s, 1H, H_{17}), 7.75 (d, 1H, J = 7.7Hz, H_{26}), 7.57 (m, 2H, H_{15} + H_{24}), 7.34 (m, 1H, H_{16} + H_{14}), 4.43 + 4.06 + 3.97 (m, 1H, H_1), 4.20 + 3.95 + 3.66 + 3.50 + 3.33 + 3.10 + 2.87 (m, 4H, 2xH_5 + 2xH_6), 3.95 + 3.80 (m, 1H overlapped, H_8), 1.70-1.11 (m, 9H, 2xH_2 + 2xH_3 + 2×H_4 + 2xH_9 + H_{10}), 0.90-0.82 (m, 6H, 3×H_{11} + 3xH_{12}) ppm.

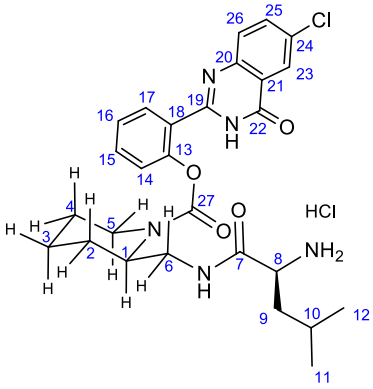
^{13}C-NMR (D_2O, 125 MHz): δ = 170.3 (C_7), 163.9 (C_{22}), 153.9 (C_{19}), 151.1 + 151.0 (C_{27} + C_{13}), 146.9 (C_{20}) 136.3 (C_{25}), 133.1 + 133.0 (C_{15}), 131.6 (C_{16}), 129.7 (C_{17}), 128.7 (C_{24}), 126.8 (C_{18}), 126.3 (C_{23}), 125.6 + 125.5 + 125.4 (C_{26}), 125.0 + 124.8 (C_{14} + C_{18}), 119.8 (C_{21}), 52.0 + 52.9 + 58.8 (C_8), 51.4 + 51.1 + 50.8 (C_1), 40.5 + 40.4 + 40.1 + 40.0 + 39.9 + 38.2 + 38.1 (C_5 + C_6 + C_9), 25.7 + 25.5 + 24.9 + 24.8 (C_2 + C_4), 24.0 + 23.9 (C_{10}), 21.8 + 21.0 + 20.9 (C_{11} + C_{12}), 18.2 + 18.1 + 18.0 (C_3) ppm.

HRMS: $C_{27}H_{33}ClN_5O_4$ $[M+H]^+$ m/z found: 526.2199 calc. 526.2216

R_f = 0.47 (DCM / MeOH 9 : 1 v/v) – revealed by UV and ninhydrine

1c: (S)-2-(6-chloro-4-oxo-3,4-dihydroquinazolin-2-yl)phenyl 2-((2-amino-4-methylpentanamido)-methyl)piperidine-1-carboxylate hydrochloride

14c (400 mg, 0.60 mmol), 2M HCl in Et_2O (3 mL, 6.0 mmol), in dry DCM (3 mL) were used to produce **1c** (300 mg, 0.53 mmol) in 88 % yield.



NMR: ^{1}H-NMR (CD_3OD, 500 MHz) δ = 8.20 (s, 1H, H_{23}), 7.91 (d, 1H, J = 7.6Hz, H_{25}), 7.81 (d, 1H, J = 7.6Hz, H_{17}), 7.77 (t, 1H, J = 7.6Hz, H_{26}), 7.71 (t, 1H, J = 7.6Hz, H_{15}), 7.53 + 7.48 + 7.40 (m, 2H, H_{16} + H_{14}), 4.60 + 4.43 (br s + m, 1H, H_1), 4.24 + 3.85 + 3.75 + 3.50 + 3.38 + 3.18 + 2.95 (m, 4H, 2xH_5 + 2xH_6), 3.90 + 3.70 (m, 1H overlapped, H_8), 1.66-1.22 (m, 9H, 2xH_2 + 2xH_3 + 2×H_4 + 2xH_9 + H_{10}), 0.90 + 0.82 (m, 6H, 3×H_{11} + 3xH_{12}) ppm.

^{13}C-NMR (CD_3OD, 125 MHz): δ = 171.1 + 171.0 + 170.7 (C_7), 161.5 + 161.3 + 161.1 + 161.0 (C_{22}), 156.6 + 156.4 (C_{19}), 154.5 + 154.3 + 154.0 + 153.9 (C_{27}), 150.5 + 150.4 (C_{13}), 142.7 + 142.5 + 142.2 (C_{20}) 137.3 (C_{25}), 135.6 + 135.4 + 134.3 (C_{15}), 131.4 (C_{17}), 127.4 (C_{16} + C_{23}), 126.2 + 126.0 + 125.9 + 125.8 (C_{26}), 124.8 + 124.7 + 124.6 + 124.4 (C_{14} + C_{18}), 122.6 + 122.5 (C_{21} + C_{24}), 53.1 + 53.0 (C_8), 52.7 + 52.6 + 52.3 + 52.2 (C_1), 41.7 + 41.6 + 41.5 + 41.4 + 41.2 + 41.1 + 39.8 + 39.4 + 39.2 (C_5 + C_6 + C_9), 27.2 + 27.0 + 26.8 + 26.4 + 26.0 (C_2 + C_4), 25.4 + 25.3 (C_{10}), 23.2 + 22.0 (C_{11} + C_{12}), 19.9 + 19.7 + 19.5 (C_3) ppm.

HRMS: $C_{27}H_{33}ClN_5O_4$ $[M+H]^+$ m/z found: 526.2214 calc. 526.2216

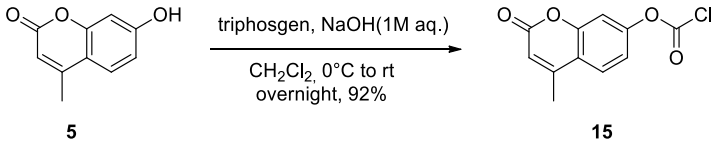
R_f = 0.40 (DCM / MeOH 95 : 5 v/v) – revealed by UV and ninhydrine

1d: (S)-2-(4-oxo-3,4-dihydroquinazolin-2-yl)phenyl 2-((2-amino-4-methylpentanamido)methyl)-piperidine-1-carboxylate hydrochloride

14d (300 mg, 0.50 mmol), 2M HCl in Et_2O (3 mL, 6.0 mmol), in dry DCM (3 mL) were used to produce **1d** (259 mg, 0.49 mmol) in 98 % yield.

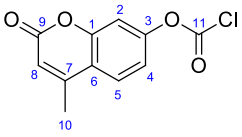
NMR and mass analysis matches the ones previously described by our group.[1]

1.6 Synthesis of chlorofomate of 4-methylumbelliferone (15)



15: 4-methyl-2-oxo-2H-chromen-7-yl carbonochloridate

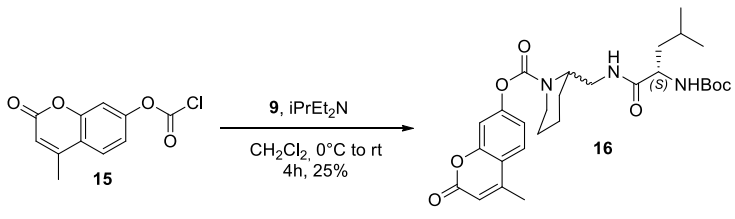
To an ice-cold solution of triphosgene (420 mg, 1.4 mmol, 0.7 eq) in DCM (10 mL) was added 4-methylumbelliferone **5** (360 mg, 2.0 mmol) in one portion. To this white suspension was added dropwise an aqueous solution of NaOH (2M, 1.1 mL, 2.2 mmol, 1.1 eq) and the reaction mixture was stirred at 0 °C for 1 h and at RT overnight. The evolution of the reaction can be monitored by TLC (cyclohexane / ethyl acetate 6:4 v/v). The suspension quickly turns to a yellow slurry before becoming clear. The next morning, a white precipitate has appeared. This precipitate was filtered, washed twice with DCM, and evaporated to dryness under reduced pressure to give about half of the expected amount of 15 (202 mg, 0.85 mmol). The filtrate was washed twice with water and the organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure to give another batch of 15 (240 mg, 1.0 mmol) as a white powder. The overall yield of the reaction is 93 %. This compound was used in the next step without further purification.



NMR: ^{1}H-NMR (300 MHz, $CDCl_3$): δ= 7.69-7.62 (m, 1H, H_5), 7.33-7.19 (m, 2H, H_2 + H_4), 6.31 (s, 1H, H_8), 2.46 (s, 3H, 3xH_{10}) ppm.

R_f = 0.47 (cyclohexane / ethyl acetate 6 : 4 v/v)) – revealed by UV

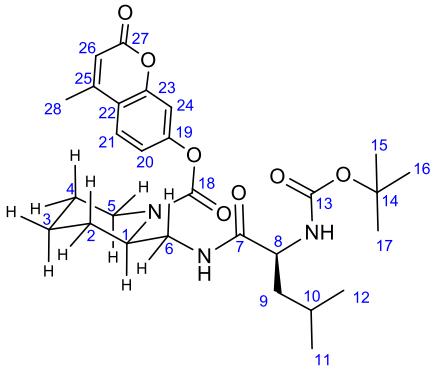
1.7 Synthesis of protected fluorogenic probe releasing umbelliferone (16)



16: (S)-4-methyl-2-oxo-2H-chromen-7-yl 2-((2-((tert-butoxycarbonyl)amino)-4-methylpentanamido)-methyl)piperidine-1-carboxylate

To an ice-cold solution of **15** (120 mg, 0.5 mmol) in anhydrous DCM (5 mL) under argon was added dropwise a solution of **9** (180 mg, 0.55 mmol, 1.1 eq) in anhydrous DCM (5 mL), followed by addition

of diisopropylethylamine (175 µL, 1.0 mmol, 2.0 eq). The reaction mixture was stirred at 0 °C for 1 h and at RT for another 3 h. The reaction can be monitored by TLC (cyclohexane / ethyl acetate 6:4 v/v). At the end of the reaction, the mixture was diluted in DCM (20 mL) and extracted twice with $NaHCO_3$ sat. (2x30 mL). The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure to give the crude product as a white solid. Two consecutive purifications via column chromatography on silicagel with cyclohexane / ethyl acetate 5:5 v/v as eluent were necessary to get rid of the unreacted fluorophore and gave **16** as a white foamy solid (65 mg, 0.13 mol) in 25% yield.



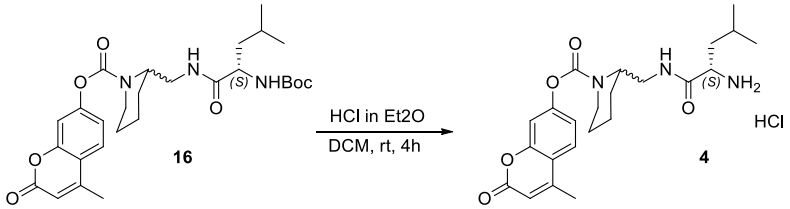
NMR: ^{1}H-NMR (500 MHz, $CDCl_3$): δ= 7.57-7.55 (2d, 1H, H_{21}), 7.16-7.11 (m, 2H, H_{20} + H_{24}), 6.49+6.40 (2 br s, 1H, NH_{amide}), 6.24 (br s, 1H, H_{26}), 4.86+4.80 (2 br s, 1H, $NH_{carbamate}$), 4.50+4.43 (2 br s, 1H, H_1), 4.10-4.15 (m, 1H, H_{5a}), 4.05 (br s, 1H, H_8), 3.93-3.22 (m, 2H, H_{6a} + H_{6b}), 3.22-2.9 (m, 1H, H_{5b}), 2.41 (s, 3H, 3xH_{28}), 1.74-1.60 (m, 7H, H_{2a} + 2xH_3 + 2xH_4 + H_{9a} + H_{10}), 1.56-1.49 (m, 1H, H_{2b}), 1.40 (br s, 10H, 3×H_{15} + 3×H_{16} + 3×H_{17} + H_{9b}), 0.90-0.85 (m, 6H, 3xH_{11} + 3xH_{12}) ppm.

^{13}C-NMR (125 MHz, $CDCl_3$): δ= 173.1 (C_7), 160.8 (C_{27}), 155.8 (C_{19}), 154.3 (C_{13}), 154.2 + 154.1 (C_{25} + C_{23}), 152.1 (C_{18}), 125.3 + 125.2 (C_{21}), 118.4 + 118.3 (C_{20}), 117.4 + 117.3 (C_{22}), 114.3 (C_{26}), 110.5 + 110.4 (C_{24}), 90.1 (C_{14}), 52.3 (C_8), 51.3 (C_1), 41.4 (C_9), 40.7 (C_5), 39.3 (C_6), 28.4 (C_{15} + C_{16} + C_{17}), 26.5 + 26.4 (C_4), 25.4 (C_2), 24.9 + 24.8 (C_{10}), 23.1 + 21.9 (C_{11} + C_{12}), 19.2 (C_3), 18.8 (C_{28}) ppm.

HRMS: $C_{28}H_{40}N_3O_7$ $[M+H]^+$ m/z found: 530.2847 calc. 530.2861

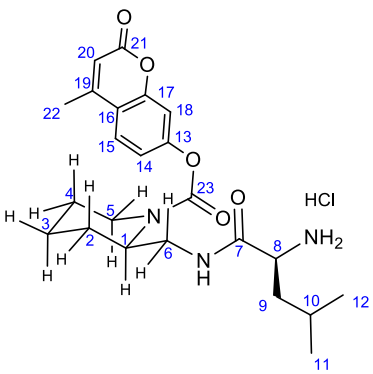
R_f = 0.33 (cyclohexane / ethyl acetate 5 : 5 v/v) – revealed by UV and ninhydrine

1.8 Deprotection toward probe releasing umbelliferone (4)



4: (S)-4-methyl-2-oxo-2H-chromen-7-yl 2-((2-amino-4-methylpentanamido)methyl)piperidine-1-carboxylate hydrochloride

This molecule was synthesized following the general synthetic procedure of 1a-d using **16** (50 mg, 0.09 mmol), 2M HCl in Et_2O (2 mL, 6.0 mmol), in dry DCM (1 mL) were used to prepare **4** (40 mg, 0.27 mmol) as a white powder in 91 % yield.



NMR: ^{1}H-NMR (500 MHz, CD_3OD): δ= 7.81 (d, 1H, J = 8.3Hz, H_{15}), 7.31-7.18 (m, 2H, H_{14} + H_{18}), 6.33 (s, 1H, H_{20}), 4.56 (m, 1H, H_1), 4.23 + 4.13 + 4.04 + 3.95 + 3.84 + 3.66 + 3.59 + 3.22 + 3.14 (m, 4H, 2xH_5 + 2xH_6), 3.99 + 3.92 + 3.80 (m, 1H overlapped, H_8), 2.50 (s, 3H, 3xH_{22}),

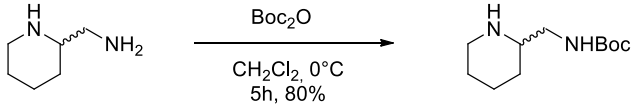
1.80-1.65 (m, 9H, 2xH_2 + 2xH_3 + 2×H_4 + 2xH_9 + H_{10}), 1.03 + 0.89 (m, 6H, 3×H_{11} + 3xH_{12}) ppm.

^{13}C-NMR (125 MHz, CD_3OD): δ= 169.4 (C_7), 161.2 (C_{21}), 154.1 (C_{19} + C_{17}), 153.8 (C_{23}), 125.7 (C_{15}), 118.1 (C_{14}), 117.3 (C_{16}), 113.2 (C_{20}), 109.8+109.7 (C_{16}), 52.5 + 52.0 + 51.6 + 51.2 + 50.8 (C_1 + C_8), 43.2 + 43.0 + 41.9 + 40.2 + 20.1 + 39.6 + 38.4 (C_5 + C_6 + C_9), 26.2 + 26.0 + 25.2 + 24.8 (C_2 + C_4), 24.1 + 24.0 + 23.9 (C_{10}), 21.8 + 20.3 (C_{11} + C_{12}), 18.7 + 18.6 (C_3), 17.7 (C_{22}) ppm.

HRMS: $C_{23}H_{32}N_3O_7$ $[M+H]^+$ m/z found: 430.2324 calc. 430.2336

R_f = 0.40 (DCM / MeOH 95 : 5 v/v) – revealed by UV and ninhydrine

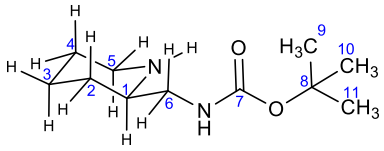
1.9 Boc Protection of the aminomethylpiperidine spacer (17)



8 **17**

17: tert-butyl (piperidin-2-ylmethyl)carbamate

To an ice-cold solution of **8** (1.154 g, 10.2 mmol 2.1 eq) in DCM (25 mL) was added dropwise over a period of 3h a solution of Boc_2O (1.094 g, 5.0 mmol) in DCM (25 mL). At the end of the addition, the white suspension was further stirred at 0 °C for 2 h and then the reaction mixture was extracted 3 times with sat. $NaHCO_3$ (3x50 mL) and once with brine (50 mL). The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure to give **17** (863 mg, 4.0 mmol) as a colorless oil which solidifies upon standing (80 % yield).



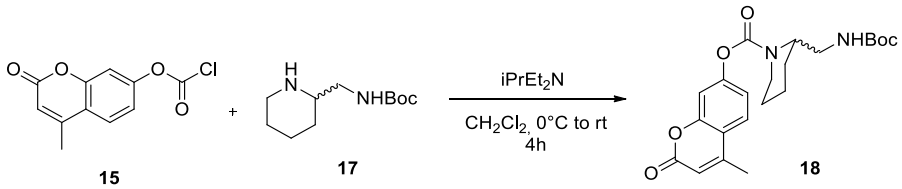
NMR: ^{1}H-NMR (500 MHz, $CDCl_3$): δ= 4.92 (br s, 1H, $NH_{carbamate}$), 3.18-3.14 (m, 1H, H_{6a}), 3.08-3.03 (br d, 1H, H_{5a}), 2.96-2.91 (q, 1H, H_{6b}), 2.65-2.58 (m, 2H, H_1 + H_{5b}), 1.81-1.76 (m, 1H, H_{4a}), 1.65-1.54 (m, 4H, NH_{amine} + H_{2a} + H_{3a} + impurities), 1.43 (s, 9H, 3×H_9 + 3×H_{10} + 3×H_{11}), 1.39-1.30 (m, 2H, H_{2b} + H_{4b}), 1.12-1.04 (m, 1H; H_{3b}) ppm.

^{13}C-NMR (125 MHz, $CDCl_3$): δ= 156.3 (C_7), 79.3 (C_8), 56.3 (C_1), 46.9 (C_6), 46.7 (C_5), 30.4 (C_3), 28.5 (C_9 + C_{10} + C_{11}), 26.6 (C_2), 24.4 (C_4) ppm.

MS: ESI: $[M+H]^+$ m/z found 215.2, calc. 215.2

R_f = 0.22 (CH_2Cl_2 / MeOH 95:5 v/v) – revealed by ninhydrine but not by UV

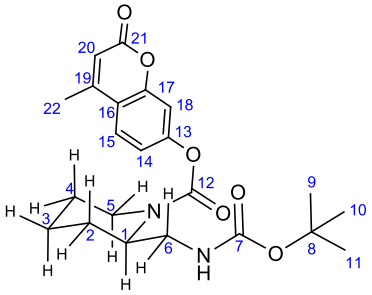
1.10 Synthesis of protected 2-component fluorophore-spacer conjugate (18)



15 **17** **18**

18: 4-methyl-2-oxo-2H-chromen-7-yl 2-(((tert-butoxycarbonyl)amino)methyl)piperidine-1-carboxylate

To an ice-cold solution of **15** (240 mg, 1.0 mmol) in anhydrous DCM (5 mL) under argon was added dropwise a solution of **17** (228 mg, 1.05 mmol, 1.05 eq) in anhydrous DCM (5 mL), followed by addition of diisopropylethylamine (350 µL, 2.0 mmol, 2.0 eq). The reaction mixture was stirred at 0 °C for 1 h and at RT for 3h. The reaction can be monitored by TLC (cyclohexane / ethyl acetate 6:4 v/v). At the end of the reaction, the reaction mixture was diluted in DCM (20 mL) and extracted twice with $NaHCO_3$ sat. (2x30 mL). The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure to give the crude product as a white solid which was purified via column chromatography on silicagel with cyclohexane / ethyl acetate 6:4 v/v as eluent to give **18** as a white solid (275 mg, 0.66 mmol) in 66 % yield.



NMR: ^{1}H-NMR (500 MHz, $CDCl_3$): δ= 7.57 (d, J = 8.6 Hz, 1H, H_{15}), 7.13 (s, 1H, H_{18}), 7.11 (d, J = 8.6 Hz, 1H, H_{14}), 6.26 (br s, 1H, H_{20}), 4.75 (br s, 1H, $NH_{carbamate}$), 4.42+3.43 (m, 1H, H_1), 4.14+3.97+3.87+3.32+2.69 (m, 2H, 2xH_5), 3.78 + 3.65 + 3.19 + 3.12 + 3.06 (m, 2H, 2xH_6), 2.43 (s, 3H, 3xH_{22}), 1.84-1.57 (m, 6H, 2xH_2 + 2xH_3 + 2xH_4), 1.53 + 1.48 + 1.43 (s, 9H, 3xH_9 + 3xH_{10} + 3xH_{11}) ppm.

^{13}C-NMR (125 MHz, $CDCl_3$): δ= 160.7, 156.0 , 154.1, 153.7, 153.5, 152.9, 152.1, 151.0, 125.2, 125.1, 118.5, 118.2, 117.9, 117.2, 114.1, 110.5, 110.3, 109.9, 82.0, 79.9, 79.7, 79.4, 53.5, 51.8, 51.1, 51.0, 47.4, 40.7, 40.5, 40.3, 40.0, 39.7, 31.4, 28.4, 28.1, 26.5, 26.4, 26.0, 25.2, 24.3, 23.2, 19.0, 18.7 ppm.

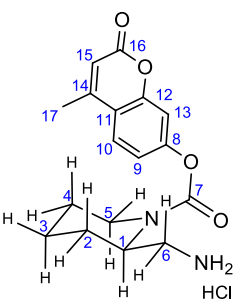
HRMS: $C_{22}H_{28}N_2O_6$ $[M+Na]^+$ m/z found: 439.1822 calc. 439.1840

R_f = 0.19 (cyclohexane / ethyl acetate 4:6 v/v) – revealed by UV and ninhydrine

1.11 Deprotection toward 2-component fluorophore-spacer conjugate (6)

6: 4-methyl-2-oxo-2H-chromen-7-yl 2-(aminomethyl)piperidine-1-carboxylate hydrochloride

This molecule was synthesized following the general synthetic procedure of **1a-d** using **18** (135 mg, 0.32 mmol), 2M HCl in Et_2O (2 mL, 6.0 mmol), in dry DCM (2 mL) were used to produce **6** (50 mg, 0.14 mmol) as a white powder in 44 % yield. This molecule is very unstable because of its propensity to cyclize, thus releasing the fluorescent coumarin. Special care should be taken when monitoring the deprotection and the reaction has been stopped by filtration as soon as the first traces of fluorophore appeared, explaining the rather low yield.



NMR: ^{1}H-NMR (500 MHz, CD_3OD): δ= 7.81 (d, J = 8.6 Hz, 1H, H_{10}), 7.27 (s, 1H, H_{13}), 7.23 (d, J = 8.6 Hz, 1H, H_9), 6.33 (s, 1H, H_{15}), 4.74+4.61 (2 br s, 1H, H_1), 4.28-4.18 (m, 1H, H_{5a}), 3.57+3.53 (m, 1H, H_{6a}), 3.25-3.17 (m, 1H, H_{5b}), 3.12+3.08 (m, 1H, H_{6b}), 2.49 (s, 3H, 3xH_{17}), 1.95-1.63 (m, 6H, 2xH_2 + 2xH_3 + 2xH_4) ppm.

^{13}C-NMR (125 MHz, CD_3OD): δ = 162.7 (C16), 155.5 (C8), 155.3 (C12), 155.0 (C14), 127.0 (C10), 119.6 (C9), 118.9 (C11), 114.7 (C15), 111.4 (C13), 51.8 + 51.3 (C1), 41.6 (C5), 40.4 (C6), 27.7 + 26.2 + 20.0 (C2 + C3 + C4), 18.7 (C17) ppm.

HRMS: $C_{17}H_{21}N_2O_4$ $[M+H]^+$ m/z found: 317.1492 calc. 317.1496

R_f = 0.19 (DCM / MeOH 9:1 v/v) – revealed by UV and ninhydrine

2 in vitro experiments

2.1 Determination of the kinetics of spacer cyclisation

10 µL of a stock solution of **6** in MeOH (10 mM) were added to wells of a 96-well black plate (Microfluor®, Thermo Scientific) containing 90 µL of aqueous buffer solution preheated to 37°C and fluorescence was recorded over the course of time by a fluorescence plate reader (37 °C, λ_{ex} = 375 nm, λ_{em}= 442 nm, Spectramax Gemini XS, Molecular Devices). The resulting curves (main article, figure 1 - left) are the mean of three simultaneous experiments (triplicates). Final probe concentration: 1 mM. Buffer used: pH 6.0, 7.0 and 7.4 (1 M phosphate buffer).

2.2 Response to enzymatic activity at high concentration

10 mM stock solutions of probes **1a-d**, **5** and **7** in DMSO were diluted with PBS (Dulbecco's Phosphate Buffer Saline, Invitrogen Corp.) to obtain a 1 mM clear solution. 10 µL aliquots of each of these solutions were added to 80 µL PBS in a 96-well black plate, and heated to 37 °C before adding 10 µL of a 35 µg.mL^{-1} solution of LAP (Leucine Aminopeptidase, microsomal from porcine kidney, Type IV-S, Sigma-Aldrich). Final concentration of probe: 100 µM; final concentration of enzyme: 3.5 µg.mL^{-1} (0.07 U.mL^{-1}). The plate was then incubated at 37 °C and fluorescence was recorded over the course of time by a fluorescence plate reader (Spectramax Gemini XS, Molecular Devices). For probes **1a-d**: λ_{ex} = 355 nm, λ_{em}= 530 nm, for probes **5** and **7**: λ_{ex} = 375 nm, λ_{em}= 442 nm. The resulting curves (Figure S1) are the mean of triplicates.

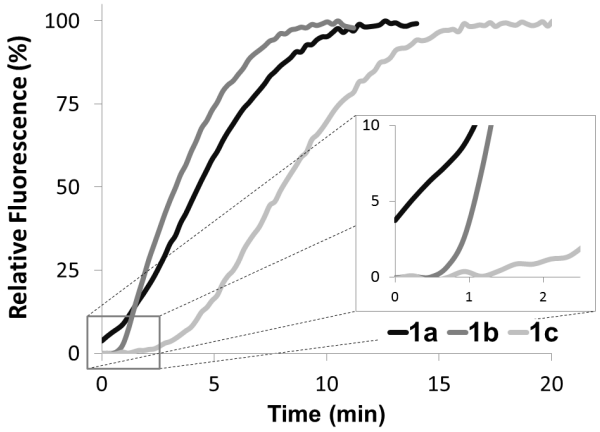


Figure S1. Influence of chlorination motif on fluorescence detection. Insert: zoom in the 3 first minutes of acquisition

2.3 Dependence of response to enzyme on probe concentration

The same protocol as the one in 2.1 was used but with variations of the quantities of probes injected and initial volumes of PBS in order to have a range of concentration between 5 µM and 100 µM (Figure 2 – right).

2.4 Fluorescence spectrum of 1a before and after enzymatic cleavage

10 µL of the above 1 mM solution of **1a** was added to 90 µL of PBS in a 96-well plate; a fluorescence emission spectrum was recorded with λ_{ex} = 355 nm (Spectramax Gemini XS, Molecular Devices).

10 µL of a 35 µg.mL^{-1} solution of LAP was then added and the fluorescence emission spectrum was recorded one more time after 2 h of reaction. Results are given in Figure S2.

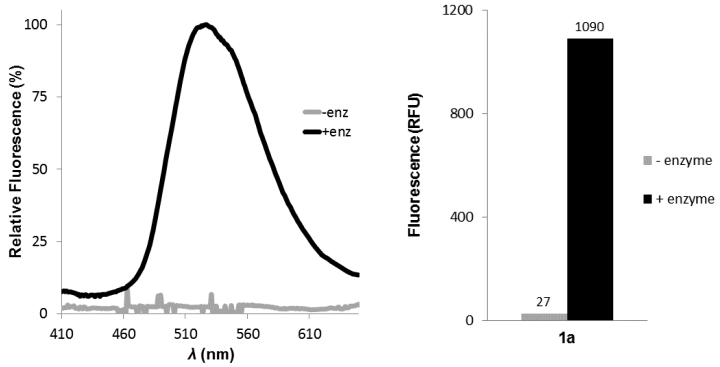


Figure S2. Left: Emission spectrum (λ_{ex}=355 nm) of probe **1a** before (grey) and after (black) enzymatic cleavage. Right: Fluorescence intensity (λ_{ex}=355 nm, λ_{em}=530 nm) before (grey) and after (black) enzymatic cleavage.

2.5 Stability of probe 1a and commercial probe AMC-Leu

To assess the stability of the probes in aqueous media at several pH values, 10 µL of a 1 mM solution of **1a** or commercial AMC-Leu **7** in PBS (containing 1% DMSO) were added to 90 µL of different buffers in a 96-well black plate. The plate was then incubated at 37 °C and fluorescence was monitored for 16 hours by a fluorescence plate reader (EnSpire, Perkin Elmer). For probes **1a**: λ_{ex} = 355 nm, λ_{em}= 530 nm, for AMC-Leu: λ_{ex} = 375 nm, λ_{em}= 442 nm. The resulting curves (Figure S3) are the mean of triplicates. Final probe concentration: 100 µM. Buffer used: pH 4.5 (20 mM citrate buffer, Sigma-Aldrich), pH 5.5 (20 mM citrate buffer, Sigma-Aldrich), pH 7.5 (20 mM phosphate buffer, Sigma-Aldrich), pH 9.5 (20 mM phosphate buffer, Sigma-Aldrich).

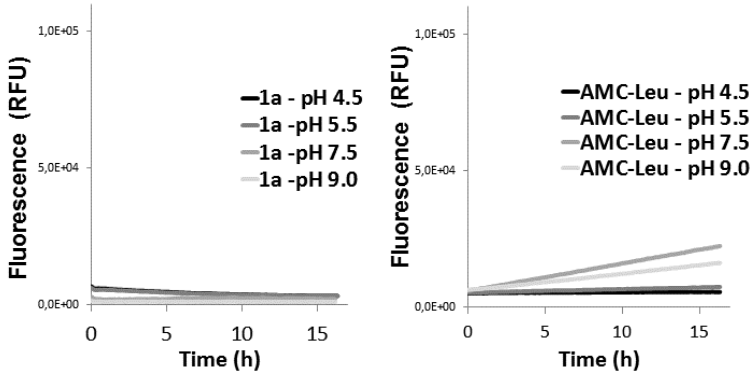


Figure S3. Stability of probe **1a** (left) and commercial AMC-Leu **7** (right) in aqueous media.

2.6 Specificity of LAP for the natural L-leucine substrate

10 mM stock solutions of probes **1a** and **1a-(R)** in DMSO were diluted with PBS to obtain a 100 µM clear solution. 10 µL of these solutions were added to 80 µL PBS in a 96-well black plate, and heated to 37 °C before adding either 10 µL of a 35 µg.mL^{-1} solution of LAP or 10 µL of PBS. Final concentration of probe: 10 µM; final concentration of enzyme: 3.5 µg.mL^{-1} (0.07 U.mL^{-1}). The plate was then incubated at 37 °C and fluorescence was recorded over the course of time by a fluorescence plate reader (EnSpire, Perkin Elmer). For probes **1a** and **1a-(R)**: λ_{ex} = 355 nm, λ_{em}= 530 nm. The resulting curves (main article, figure 3 - right) are the mean of triplicates.

2.7 Selectivity of probe 1a against several peptidases

In a similar procedure as described in paragraph 2.5, 10 µL of a 100 µM solution of **1a** in PBS (containing 0.1 % DMSO) were added to wells of a 96-well black plate containing 80 µL PBS and 10 µL of a solution of enzyme in PBS. Final concentration of probe: 10 µM; final quantity of enzymes: LAP: 0.007 U, Chymotrypsin (α-Chymotrypsin from bovine pancreas type II, Sigma-Aldrich): 5 U, Trypsin (Trypsin from bovine pancreas type I, Sigma-Aldrich): 5 U, Cathepsin B (Cathepsin B from bovine spleen, Sigma-Aldrich): 0.12 U. The plate was then incubated at 37 °C and fluorescence was monitored by a fluorescence plate reader (EnSpire, Perkin Elmer, λ_{ex} = 355nm, λ_{em}= 530nm). The resulting curves (main article, figure 3 - middle) are the mean of triplicates.

3 in cellulo experiments

3.1 Cell culture

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Corp.) supplemented with 10 % (v/v) fetal bovine serum (Invitrogen Corp.), 50 $U.mL^{-1}$ penicillin, and 50 $\mu g.mL^{-1}$ streptomycin (Invitrogen Corp.) in a humidified incubator containing 5 % CO2 in air at 37°C.

3.2 Presence of LAP in cells

3.2.1 Dosage of HeLa cells extracts with Leu-PNA

Leu-*p*NA hydrolysis was determined using a reaction mixture that contained 0 to 0.075 units of standard LAP solution from porcine kidney (blue line) or extracts of Hela cells (red points or green marker), 5 mM Leu-*p*NA in a total volume of 100 µl of Tris / $MgCl_2$ buffer (20 mM Tris-HCl pH 8.8 , 4 mM $MgCl_2$). The mixture was incubated at 37 °C and the increase in absorbance at 405 nm due to the release of *p*-nitroaniline was monitored after 15 minutes (Figure S4, EnSpire, PerkinElmer). Red points correspond to the mean of duplicates of different samples containing 7.10^4 HeLa cells and the green marker to the mean ± SEM of all the tested samples containing 7.10^4 HeLa cells , i.e. 0.7 LAP units / 10^6 HeLa cells.

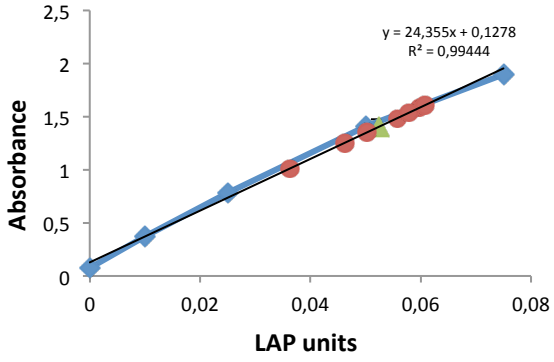


Figure S4. Dosage of LAP activity in HeLa cells

3.2.2 Zymography

HeLa cell extracts and purified LAP (microsomal from porcine kidney, Type IV-S, Sigma-Aldrich) were analyzed by native polyacrylamide gel electrophoresis (PAGE) using a 1 mm thick gel (10 x 7.5 cm, 4 % stacking gel and 7.5 % running gel) in a Mini-PROTEAN Tetra Cell PAGE apparatus (Bio-Rad Laboratories) at 120 V for 120 min at 4 °C with Tris / glycine running buffer (25 mM Tris, 195 mM glycine). After separation, the gel was incubated at 37 °C for 1 hour in 50 mM Tris-HCl buffer (pH 8.0) containing 0.5 mM $MgCl_2$ and 100 µM of either probe **1a** or commercial AMC-Leu **7**. Pictures were taken

under UV illumination (312 nm) using either a regular camera (Figure S5) or a Bio-Print Mega (Vilbert Lourmat) (Figure S6). Gels were then further stained with Coomassie Brillant Blue solution (0.05 % in H_2O / acetic acid / methanol: 65% / 10% / 25%) for 1 h and destained with a destaining solution (H_2O / acetic acid / methanol: 88% / 8% / 4%) for 2 h.

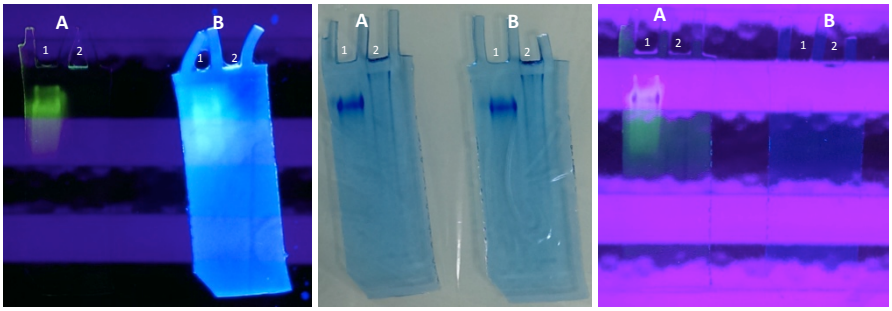


Figure S5. Images taken with a regular camera of a zymogram revealed with probe **1a** (A), AMC-Leu **7** (B). Under UV light before Coomassie staining (Left), under white light after Coomassie staining (middle), under UV light after Coomassie staining (Left), Lane 1: purified LAP; Lane 2: HeLa extracts

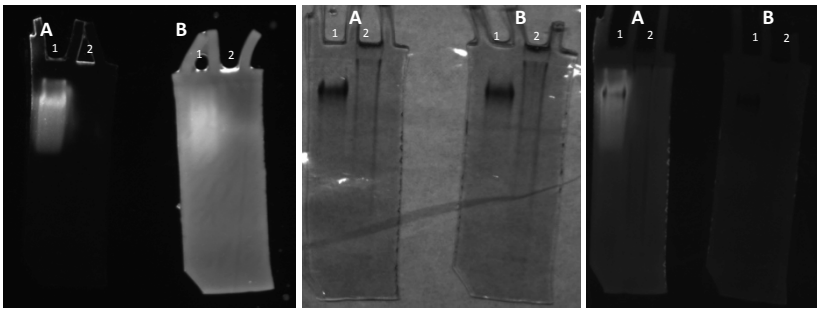


Figure S6. Images obtained with Bio-Print Mega of a zymogram revealed with probe **1a** (A), AMC-Leu **7** (B). Under UV light before Coomassie staining (Left), under white light after Coomassie staining (middle), under UV light after Coomassie staining (Left), Lane 1: purified LAP; Lane 2: HeLa extracts

3.2.3 Western Blot

Protein extracts were resolved on 8% sodium dodecyl sulphate–polyacrylamide gel, stained by coomassie blue (left panel) or transferred onto nitrocellulose membrane (right panel). The endogenous LAP protein was detected using a rabbit anti human LAP polyclonal antibody (Sigma, 1:200 dilution) and visualized using the ECL plus Western blotting detection kit (Amersham Pharmacia Biotech). For the negative control, LAP solution from porcine kidney was used. Only LAP from HeLa cells was detected as a 55-60 kD band. (Figure S7).

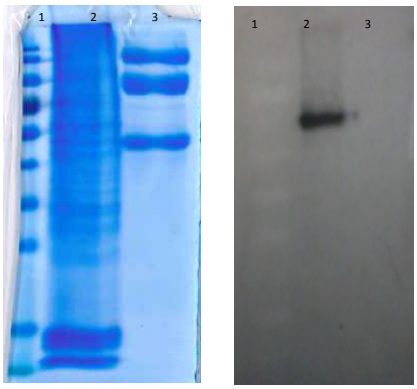


Figure S7. Left: image of the gel after electrophoresis and staining with Coomassie Brillant Blue. Right: image of the X-ray film after development of the membrane used for Western blot. Lane 1: molecular weight marker, lane 2: HeLa extracts, lane 3: purified enzyme (porcine).

3.2.4 Detection of LAP activity in HeLa cell extracts on probe-containing agar

Drops of 2 µL of HeLa extracts were placed on a 10 % agarose gel containing 100 µM of either probe **1a** or commercial AMC-Leu **7**. The presence of LAP in the extracts is revealed by the appearance of fluorescence when the gel is exposed under UV light (Figure S8).

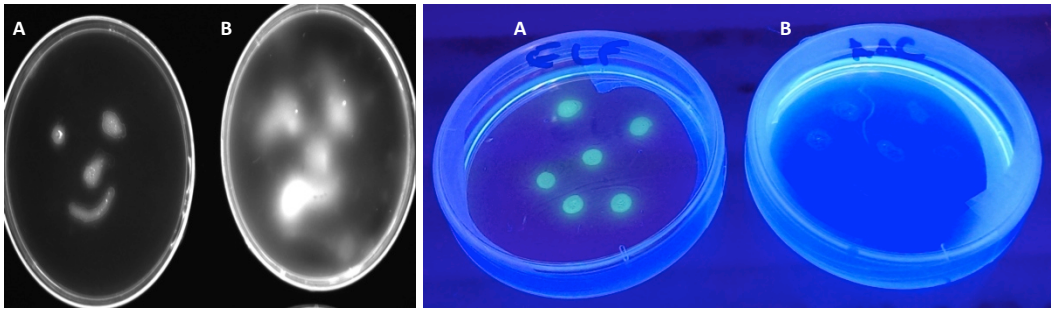


Figure S8. Agarose gel containing probe **1a** (A) or **7** (B) illuminated under UV light (312 nm), 1 h (left) or 3 days (right) after HeLa cell extract was spotted on agar surface.

3.3 Fluorescence microscopy of living cells

3.3.1 Visualization of LAP activity in HeLa cells

10^5 HeLa cells were seeded in 1 mL of supplemented DMEM in a clear 12-well plate (Corning Costar). After 24 h of incubation, the medium was removed, cells were washed with PBS and the medium was replaced by an appropriate volume of opti-MEM (Invitrogen Corp.). A desired volume of stock solutions of probes **1a** or **5** at 1 mM (in PBS, containing 1 % DMSO) or 100 µM (in PBS, containing 0.1 % DMSO) to have a range of concentrations between 5 µM and 100 µM in 1 mL final volume. Cells were then incubated for 2 h before images were taken (Figure S9 and Figure S10). The medium was then removed from the wells, cells were washed with PBS and 1 mL of fresh opti-MEM was added. A new set of images was taken (Figure S11). Note: fluorescence due to probe **1a** is still present whereas fluorescence from **5** has completely disappeared.

Fluorescence images were captured using a Zeiss AxioObserver Z1 instrument with EC Plan Neofluar 10x or a Achroplan 40× objective lens. The light source was metal halide fluorescence HPX 100. For 4-methylumbelliferone imaging (probe **5**), the Zeiss filter set 49 was used with with λ_{ex}= 335 – 383 nm and λ_{em}= 420 – 470 nm. For ELF®97 imaging (probe **1a**), the Zeiss filter set 21HE was used with λ_{ex}= 325 – 355 nm and λ_{em}= 470 – 555 nm. Images were acquired with an AxioCam MRm3 S/N 5762. Exposure time was 200 ms for both dyes and 20 ms for brightfield images.

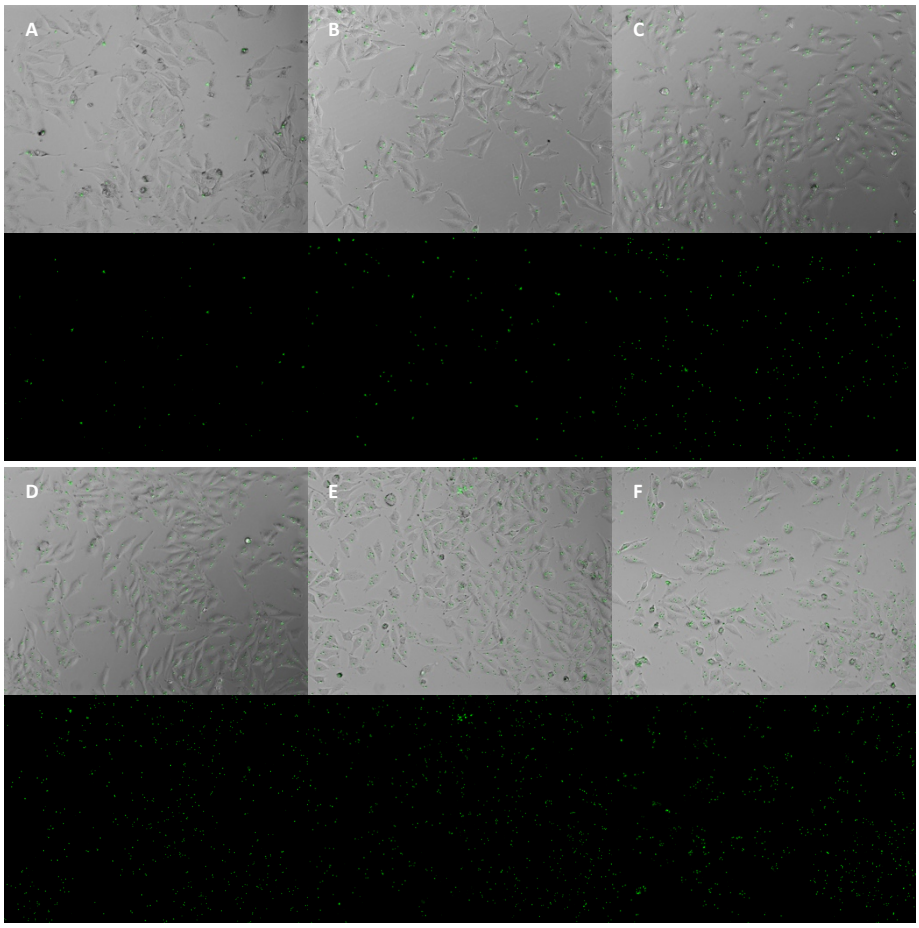


Figure S9. HeLa cells 2 hours after incubation at 37°C with increasing concentrations of probe **1a**; A: 5 µM, B: 10 µM C: 25 µM, D: 50 µM, E:75 µM, F: 100 µM; upper panels: brightfield image merged with fluorescence channel; lower panels: fluorescence channel

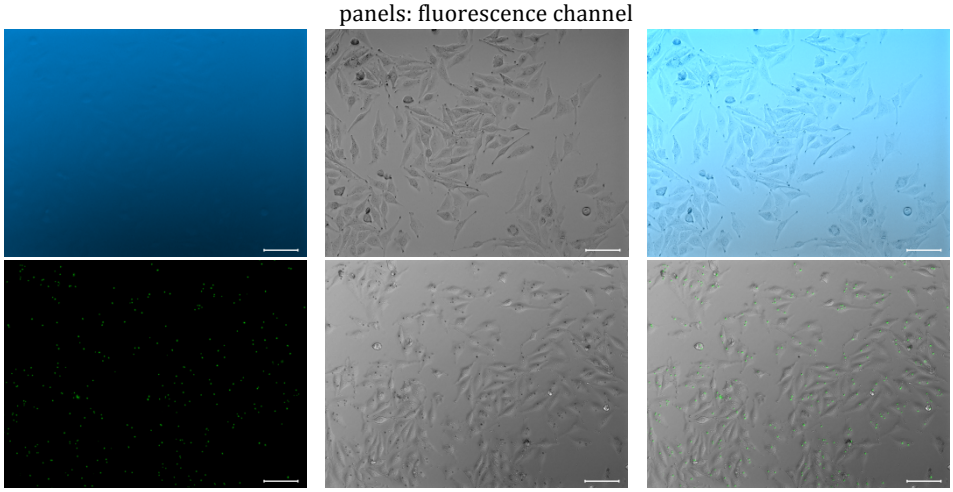


Figure S10. HeLa cells 2 hours after incubation at 37 °C with 10 µM of probe **5** (upper panels) or probe **1a** (lower panels); fluorescence image (left), bright field (middle), merged (right); scale bar: 100 µm

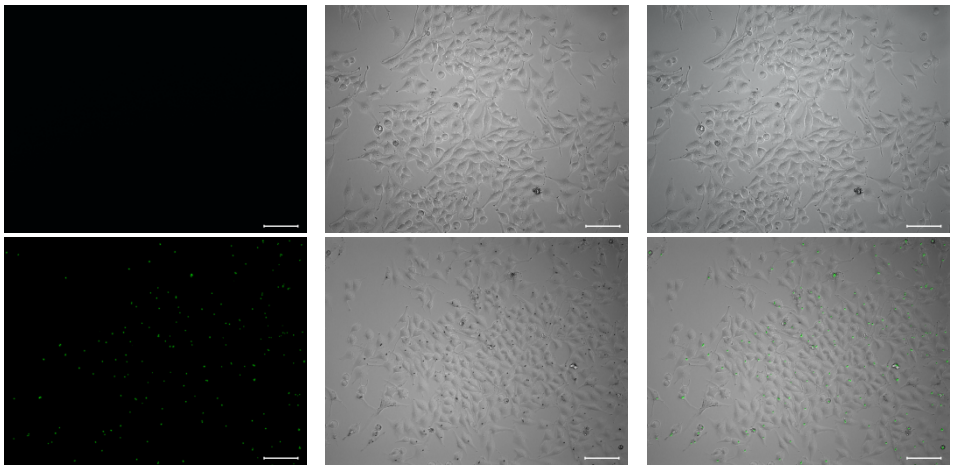


Figure S11. HeLa cells were washed 2 hours after incubation with 10 µM of probe **5** (upper panels) or probe **1a** (lower panels). Images were taken 22 h after washing; fluorescence (left), brightfield (middle), merged (right). scale bar: 100 µm

3.3.2 Time lapse experiments (movies)

For time lapse experiments, a fixed concentration of 25 µM of **1a** was used and images were acquired at RT every 10 s over 60 min (see attached movie).

3.3.3 Progressive inhibition of LAP activity in HeLa cells

5.10^4 HeLa cells were seeded in 500 µL of supplemented DMEM in a clear 24-well plate (Corning Costar). After 24 h of incubation, the medium was removed, cells were washed with PBS and medium was replaced by an appropriate volume of opti-MEM (Invitrogen Corp.). A desired volume of stock solution containing LAP inhibitors Bestatin (Sigma-Aldrich) or L-Leucinethiol (oxidized dihydrochloride form, Sigma-Aldrich) at 100 µM (in PBS, containing 0.1 % DMSO) were added to create a range of concentrations between 1 µM and 20 µM in 500 µL final volume. Cells were then incubated for 2 h before 5 µL of a 1 mM solution of probe **1a** (in PBS, containing 1% DMSO) were added to the wells (final probe concentration: 10 µM). Images (Figure S12 and Figure S13) were recorded after 1 hour of incubation.

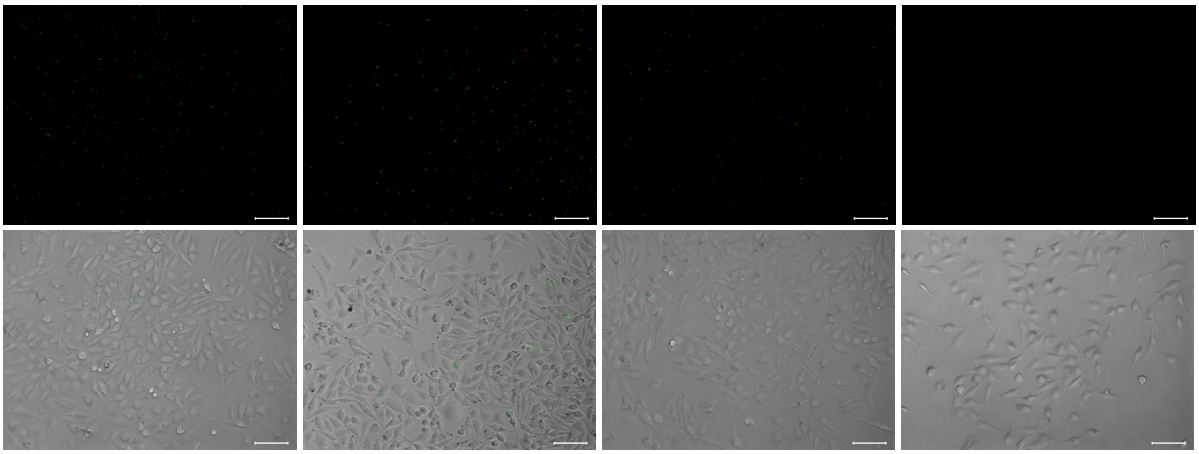


Figure S12. Pictures of HeLa cells after incubation with different concentration of bestatin (from left to right: 1 µM, 5 µM, 10 µM, 20 µM) and 10 µM of probe **1a**. Upper panels: fluorescence channel; Lower panels: merged fluorescence and brightfield images. Scale bar: 100 µm

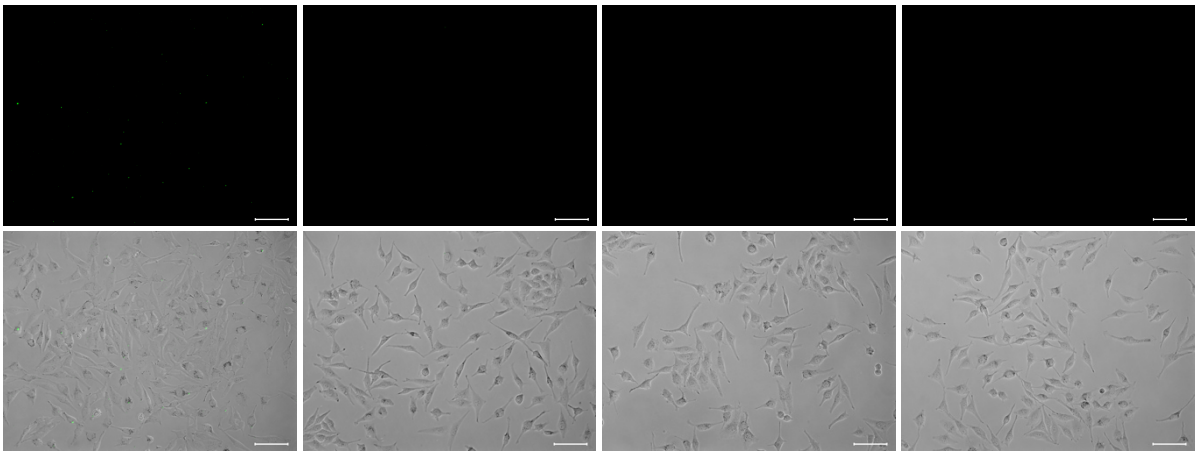


Figure S13. Pictures of HeLa cells after incubation with different concentration of L-Leucinethiol (from left to right: 1 µM, 5 µM, 10 µM, 20 µM) and 10 µM of probe **1a**. Upper panels: fluorescence channel; Lower panels: merged fluorescence and brightfield images. Scale bar: 100 µm

3.3.4 Incubation with the non-natural D-Leucine isomer 1a-(R)

5.10^4 HeLa cells were seeded in 500 µL of supplemented DMEM in a clear 24-well plate (Corning Costar). After 24 h of incubation, the medium was removed, cells were washed with PBS and medium was replaced by 495 µL of opti-MEM (Invitrogen Corp.). 5 µL of a 1 mM solution of probes **1a** or **1a-(*R*)** (in PBS, containing 1 % DMSO) were added to the wells (final probe concentration: 10 µM). Images (Figure S14) were recorded after 1 hour of incubation.

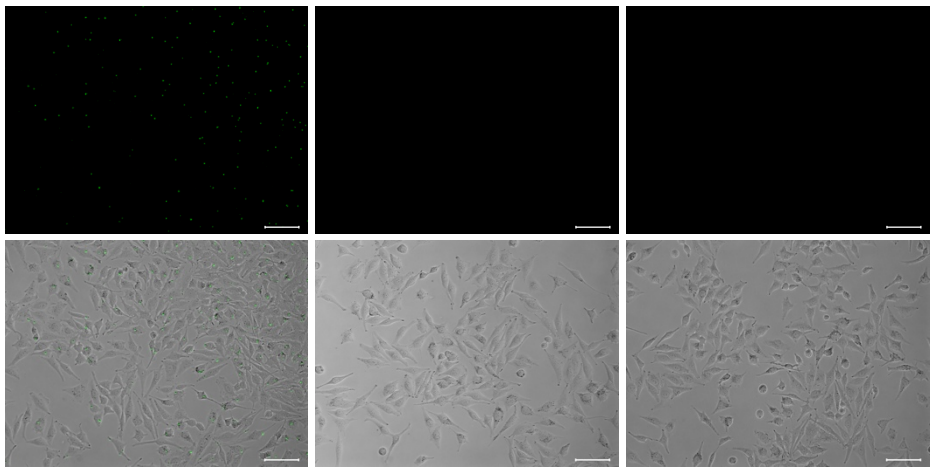


Figure S14. Pictures of HeLa cells incubated with probe **1a** (left), probe **1a-(R)** (middle) or without probe (right) Upper panels: fluorescence channel; Lower panels: merged fluorescence and brightfield images. Scale bar: 100 µm

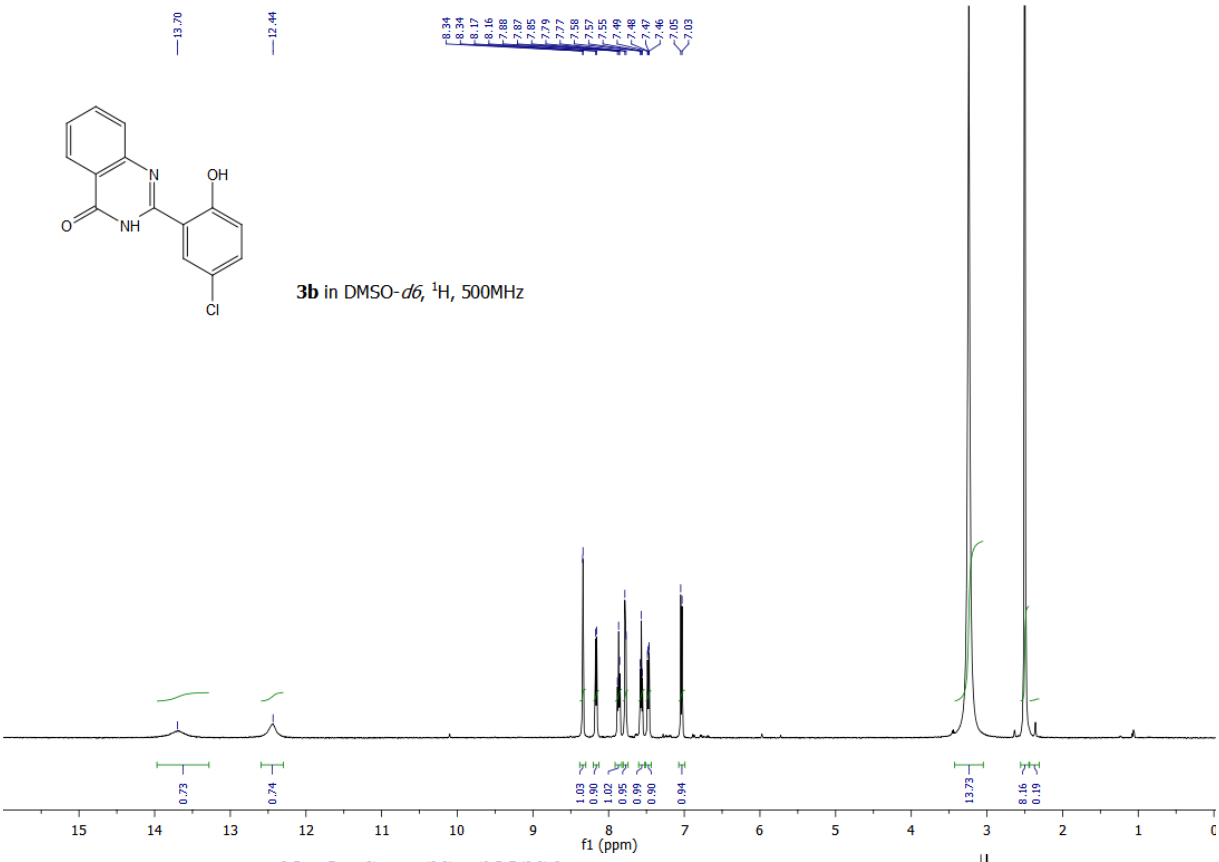
3.4 Viability tests

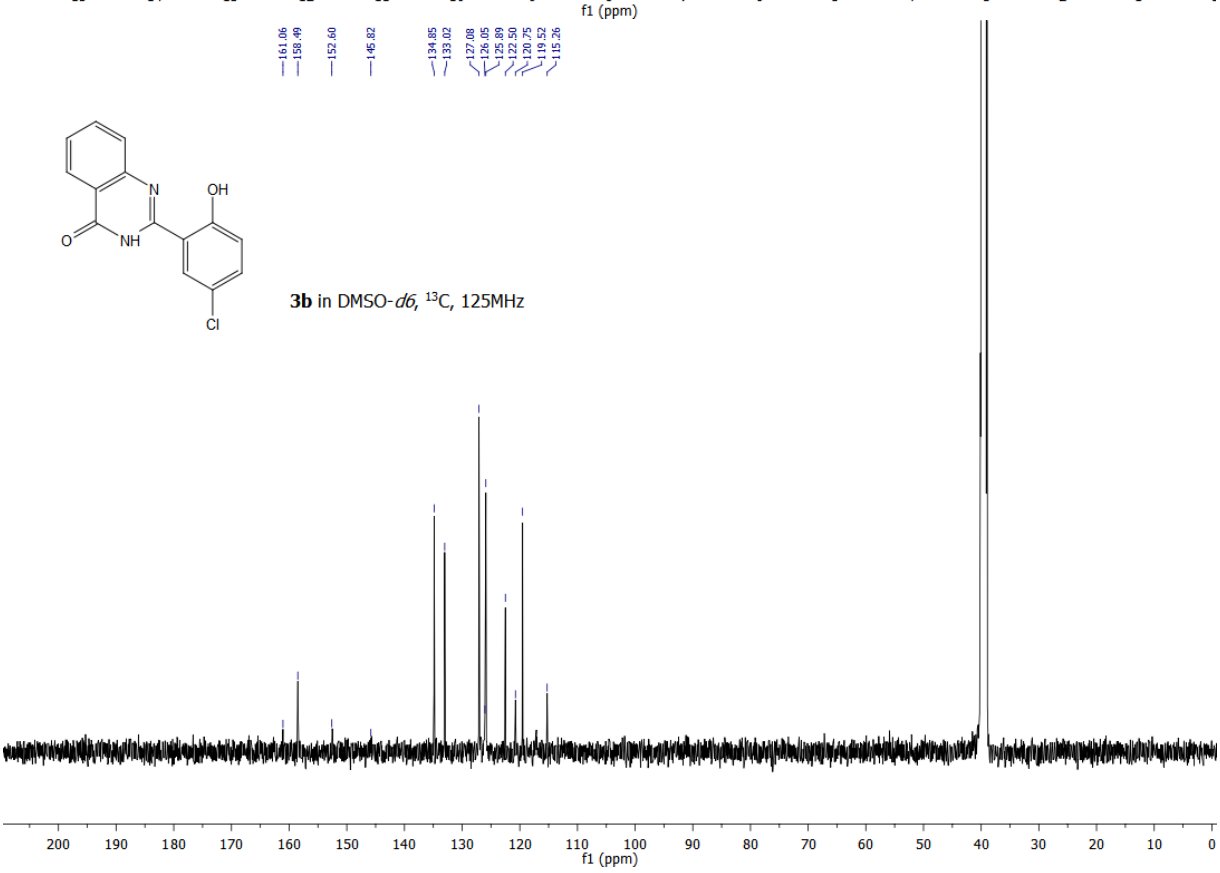
8.10^3 HeLa cells were seeded in 100 µL of supplemented DMEM in a clear 96-well plate (Corning Costar). After 24 h of incubation the medium was removed, cells were washed with PBS and medium was replaced by an appropriate volume of opti-MEM (Invitrogen Corp.). A desired volume of stock solutions of probes **1a** or **5** at 1 mM (in PBS, containing 1% DMSO) or at 100 µM (in PBS, containing 0.1 % DMSO) to create a range of concentrations between 0 µM and 100 µM in 100 µL final volume. Cells were then incubated for 24 h before 20 µL of the MTS reagent (CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay, Promega) were added. Cells were incubated for another 1.5 h period and absorbance at 493 nm was measured with a microplate reader (Multiscan GO Microplate Spectrophotometer, Thermo Scientific).

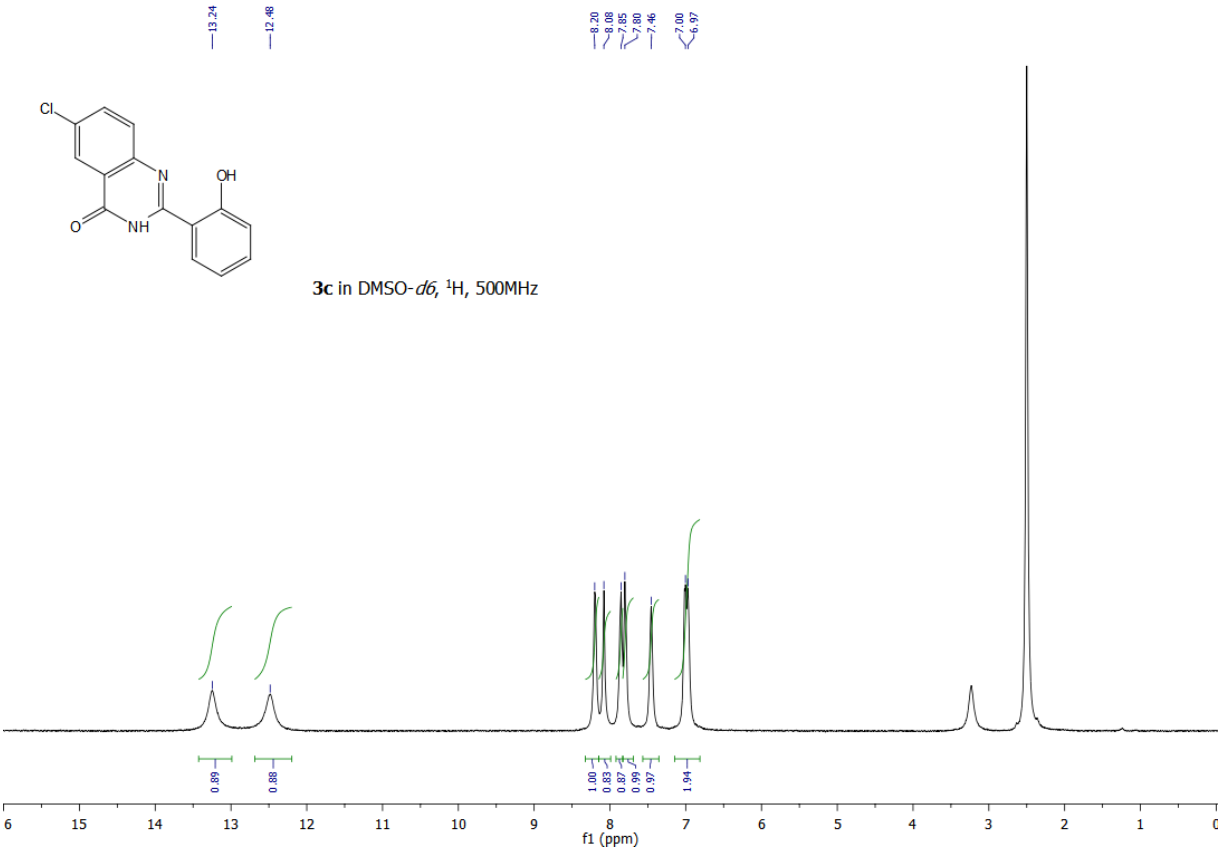
The percentage of viable cells is calculated by dividing the absorbance at a given concentration by the absorbance at 0 µM. The given results are the mean of 3 independent experiments performed in triplicates.

4 NMR Spectra

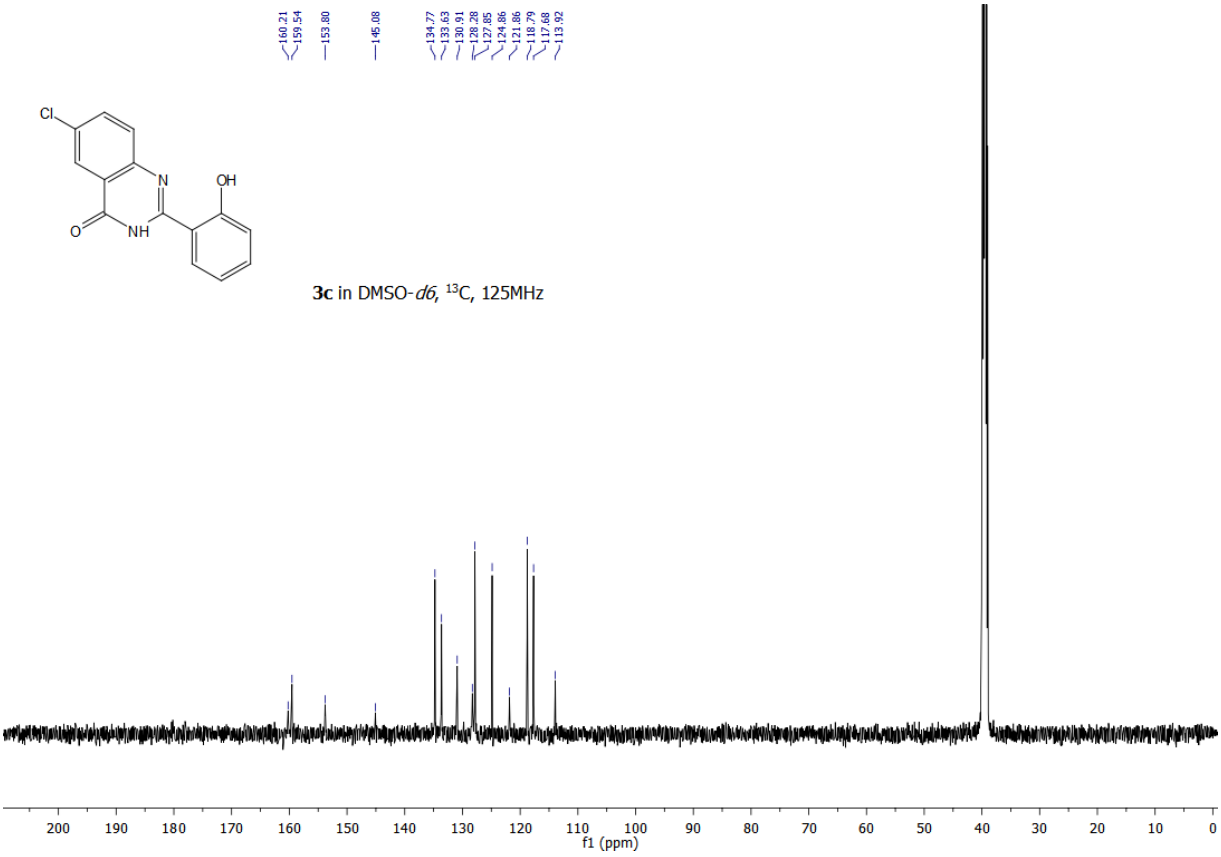
As a preamble, it should be noted that NMR spectra are very complex because of the presence of diastereoisomers (racemic aminomethylpiperidine spacer), conformers (piperidine chair) and rotamers (carbamate units). To confirm the structures, 2D experiments (COSY, HSQC, HMBC) were performed.


13.70
12.44
8.34
8.34
8.17
8.16
7.88
7.87
7.85
7.79
7.77
7.58
7.57
7.55
7.49
7.48
7.47
7.46
7.05
7.03
N
OH
O
NH
Cl
3b in DMSO-d6, 1H, 500MHz
0.73
0.74
1.03
0.90
1.02
0.95
0.99
0.90
0.94
13.73
8.16
0.19
f1 (ppm)

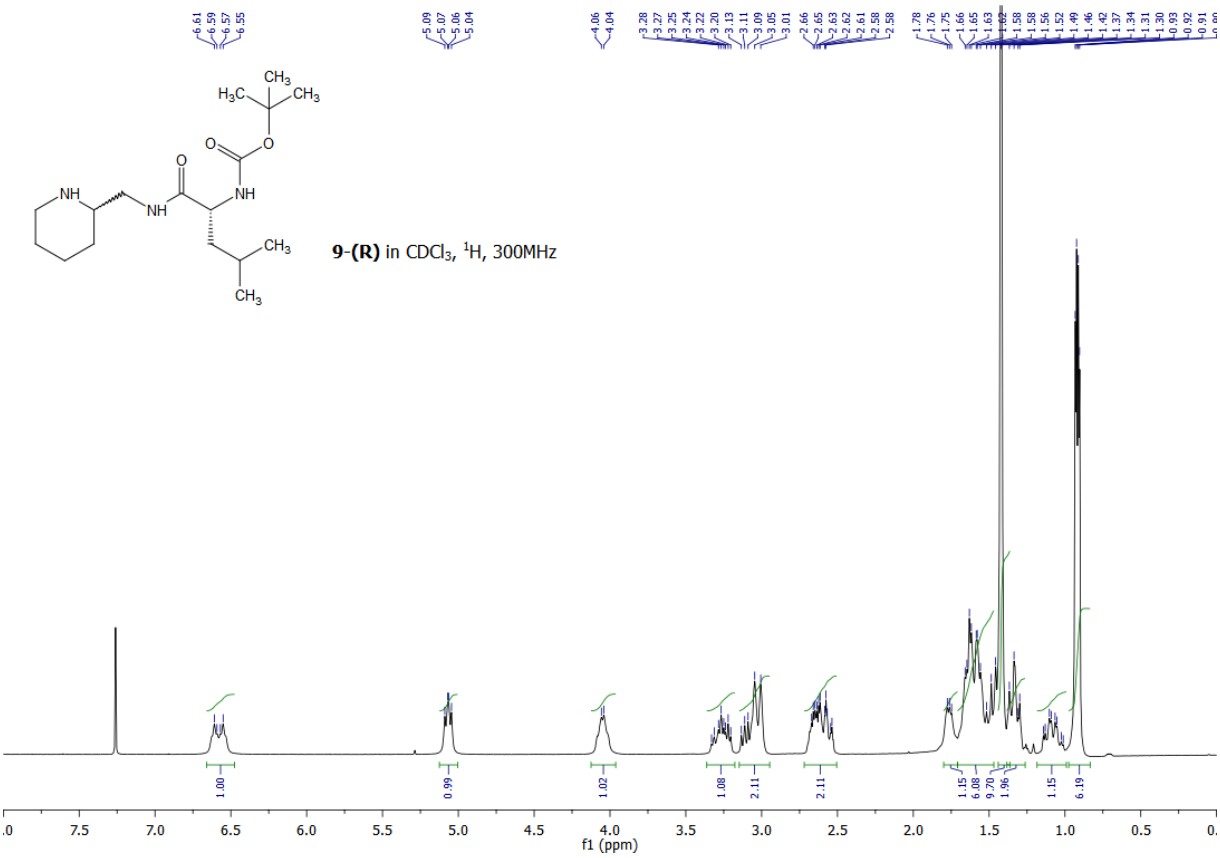

161.06
158.49
152.60
145.82
134.85
133.02
127.08
126.05
125.89
122.50
120.75
119.52
115.26
N
OH
O
NH
Cl
3b in DMSO-d6, 13C, 125MHz
f1 (ppm)

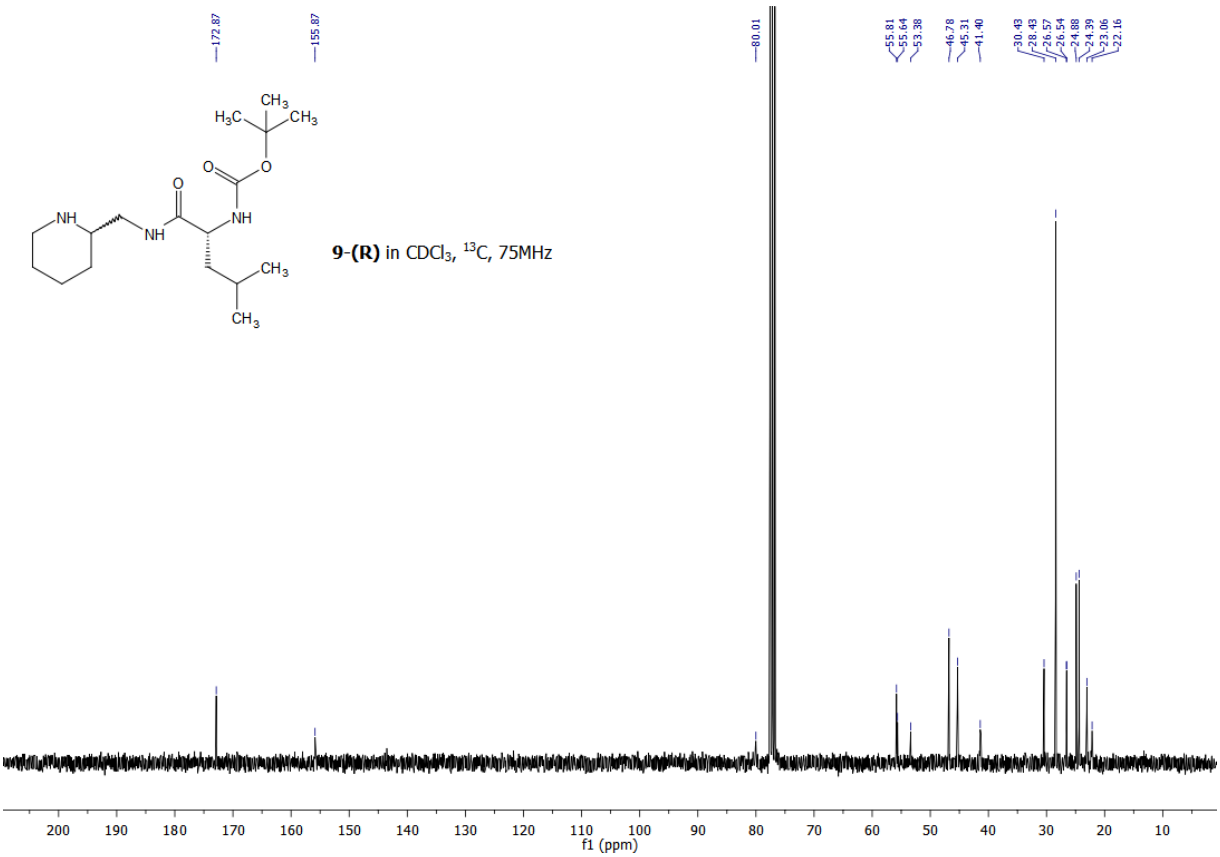


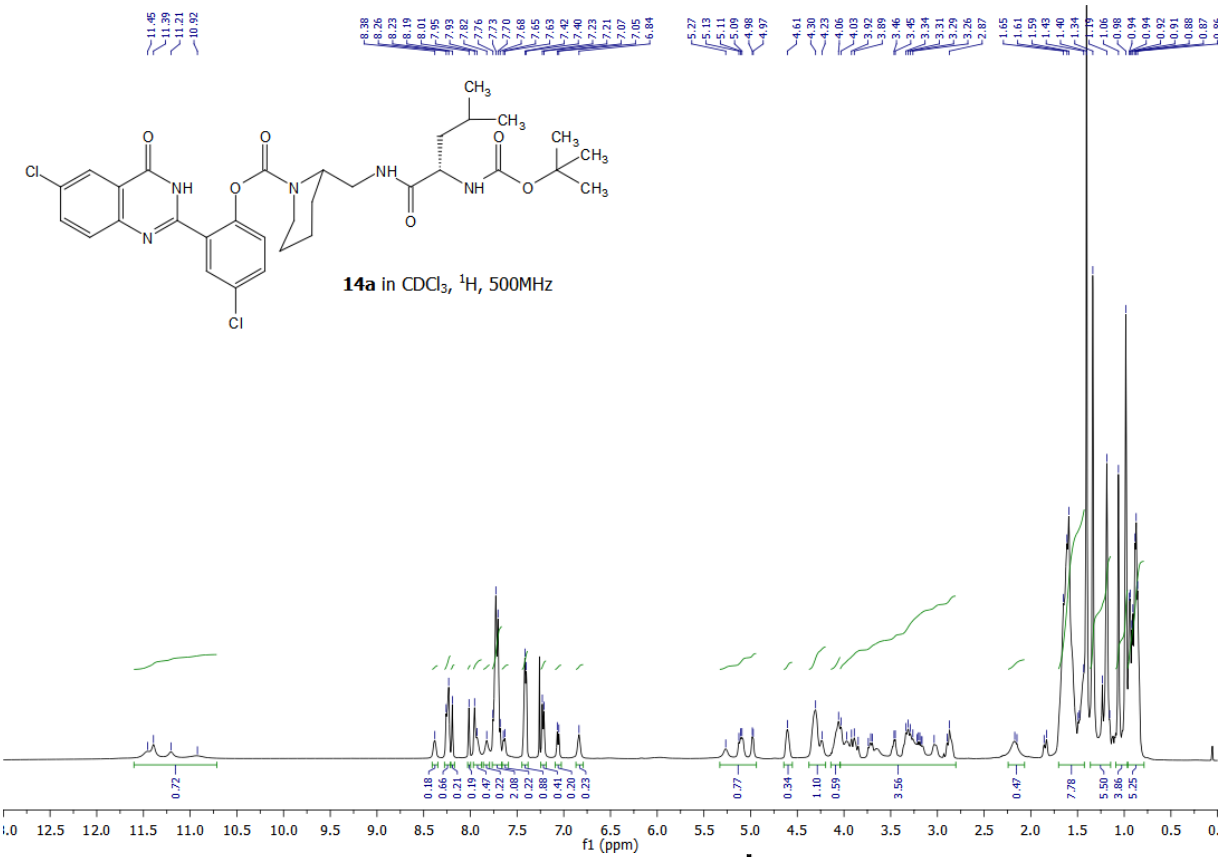
3c in DMSO-*d6*, ^{1}H, 500MHz

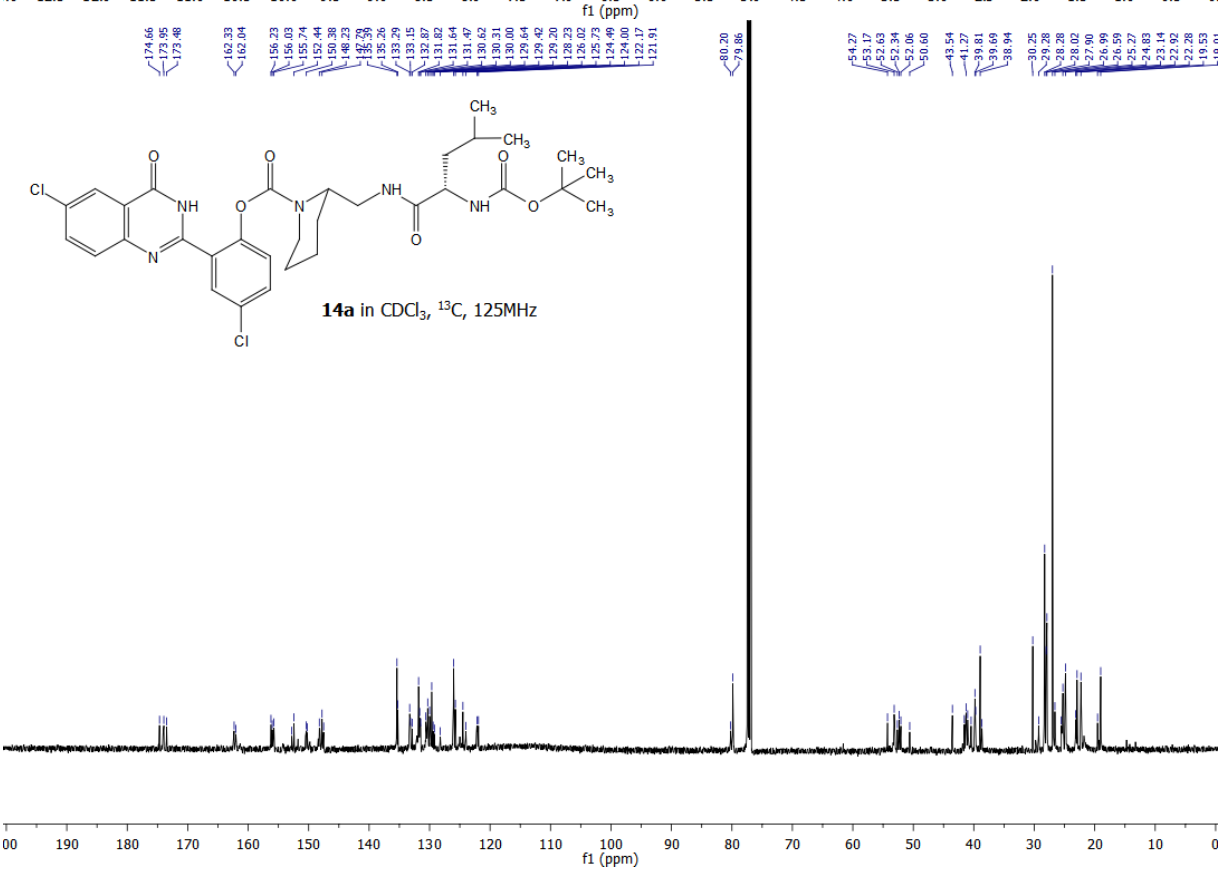


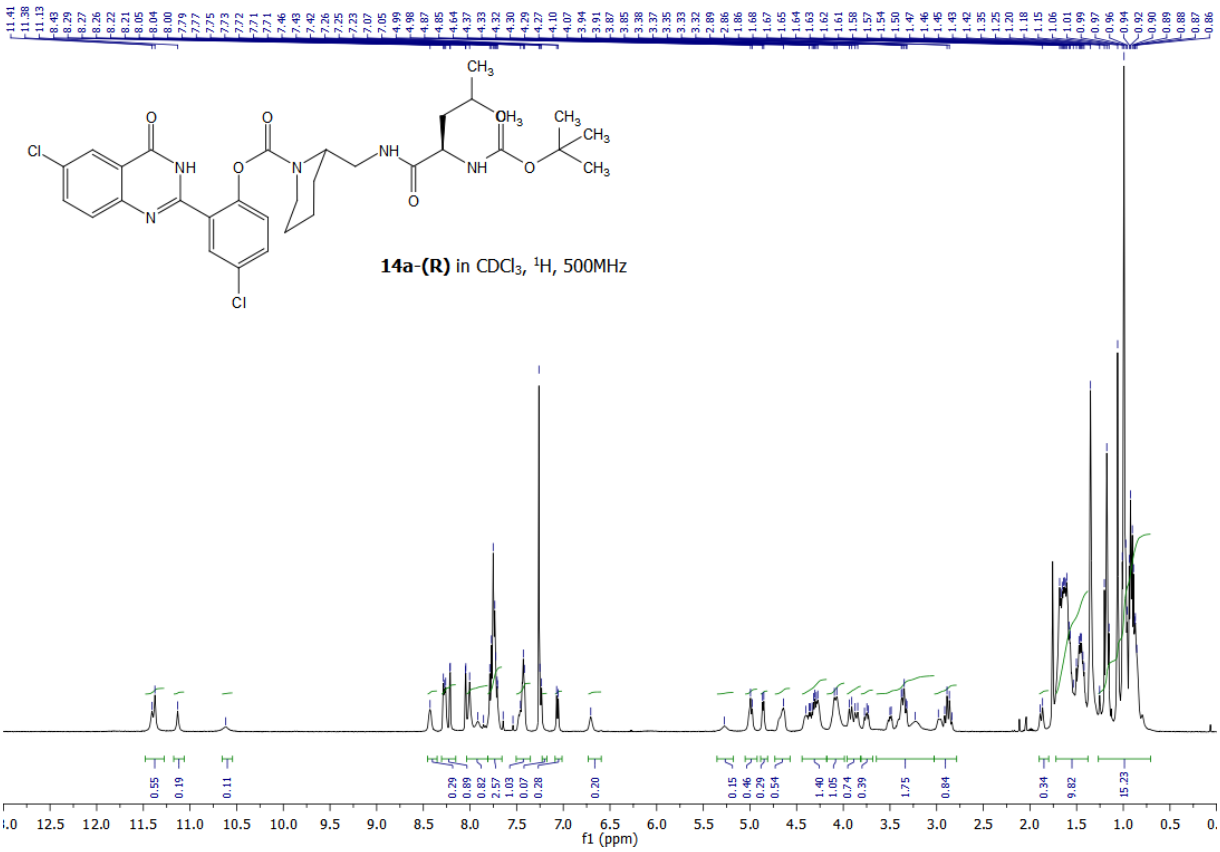
3c in DMSO-*d6*, ^{13}C, 125MHz

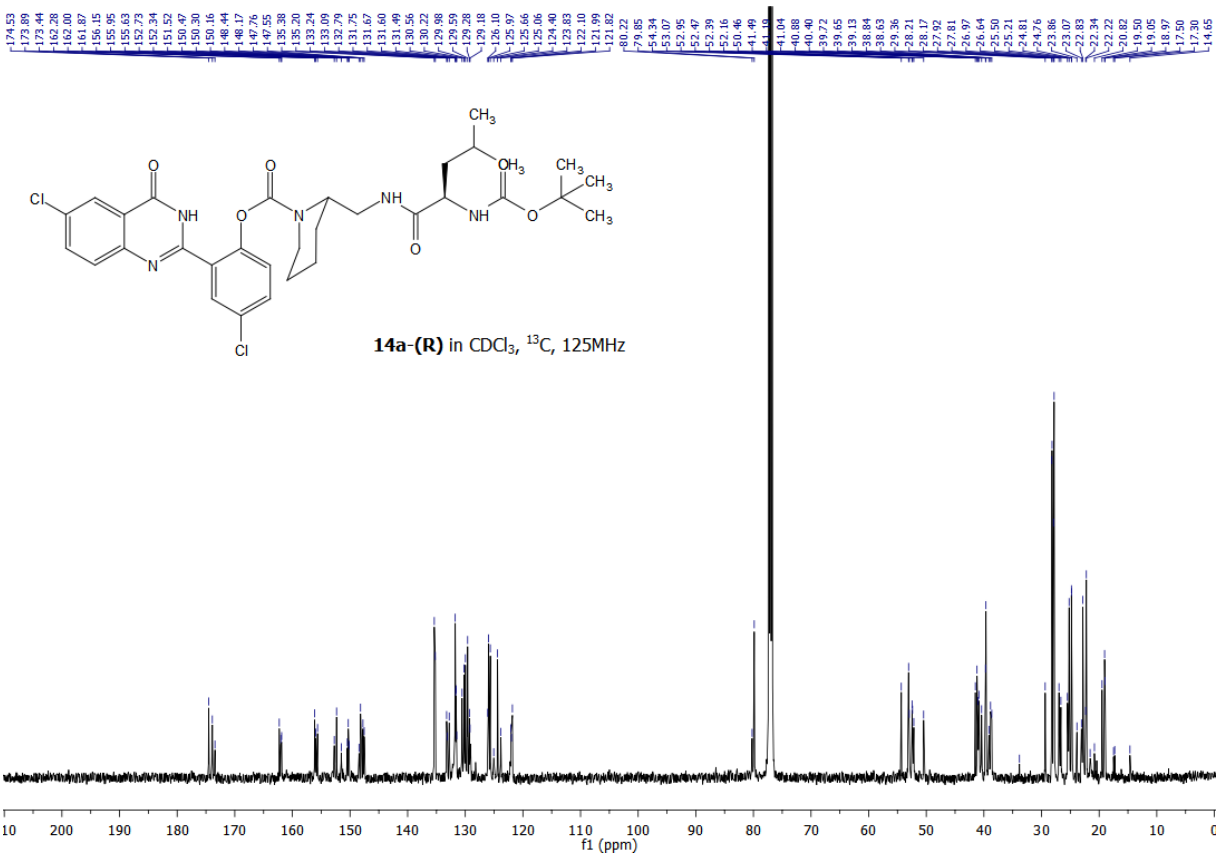

9-(R) in CDCl3, 1H, 300MHz
f1 (ppm)


9-(R) in CDCl3, 13C, 75MHz
f1 (ppm)


14a in CDCl3, 1H, 500MHz
f1 (ppm)


14a in CDCl3, 13C, 125MHz
f1 (ppm)


14a-(R) in CDCl3, 1H, 500MHz
f1 (ppm)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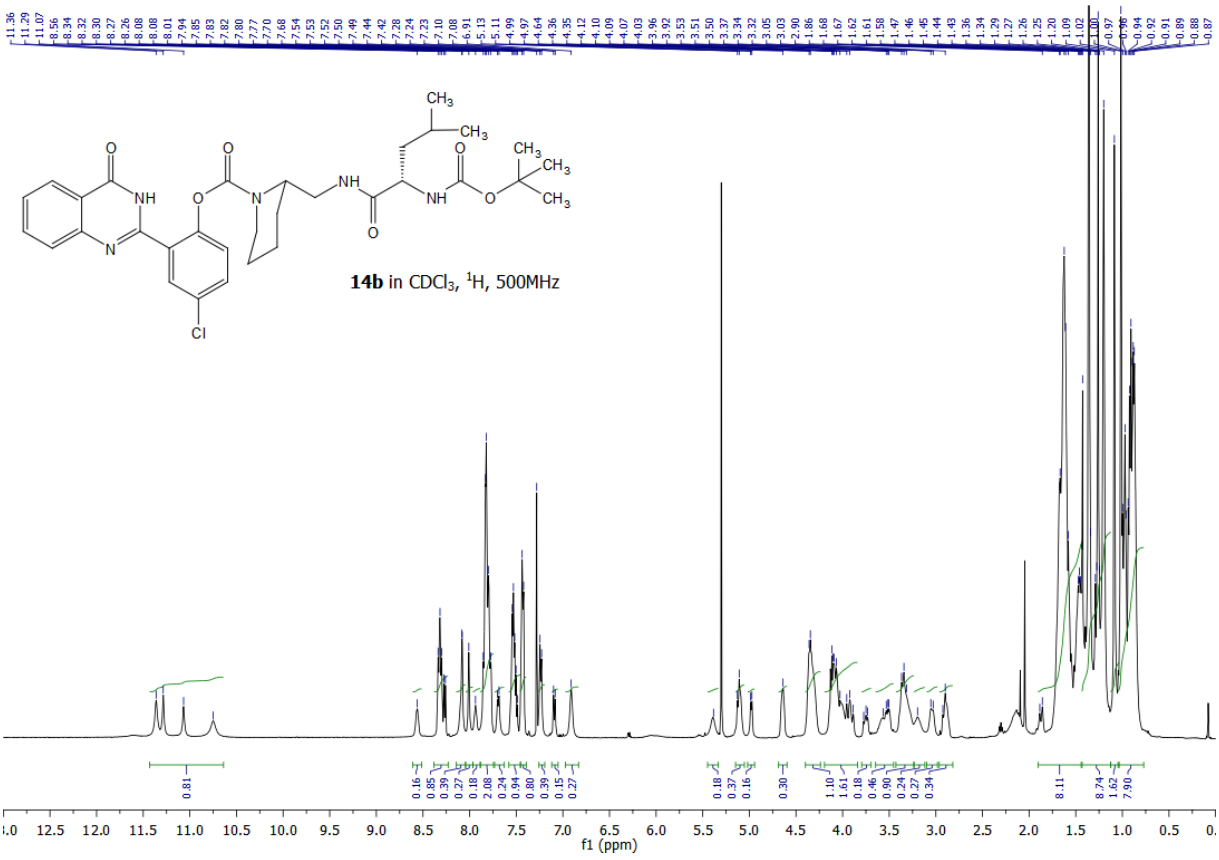
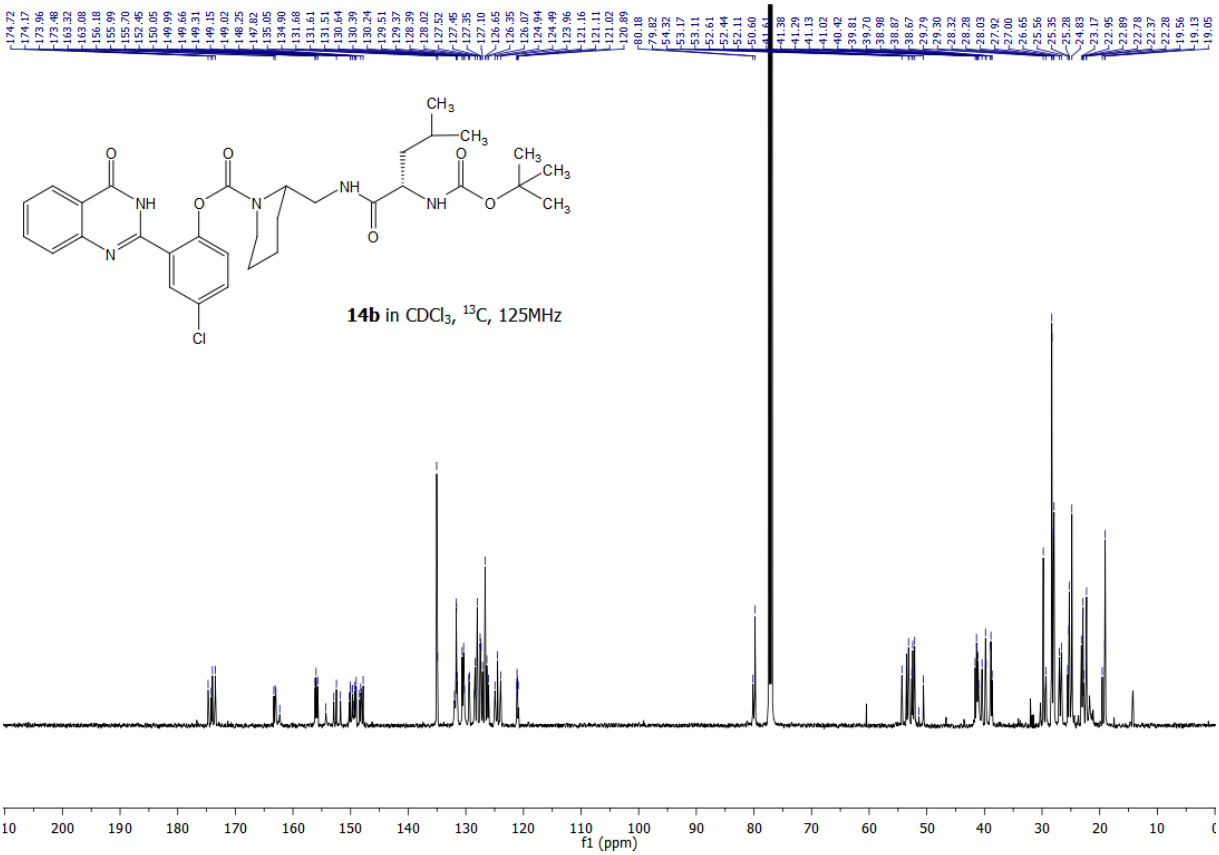
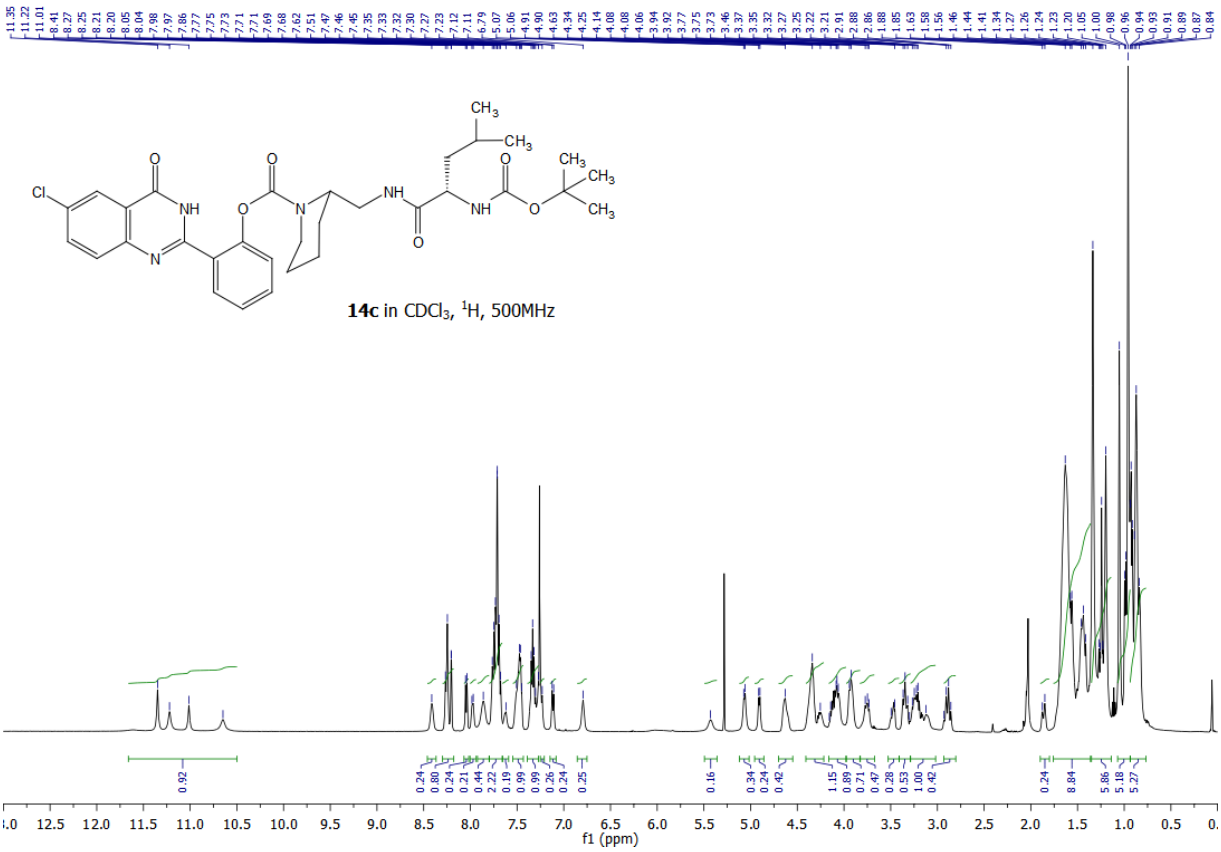
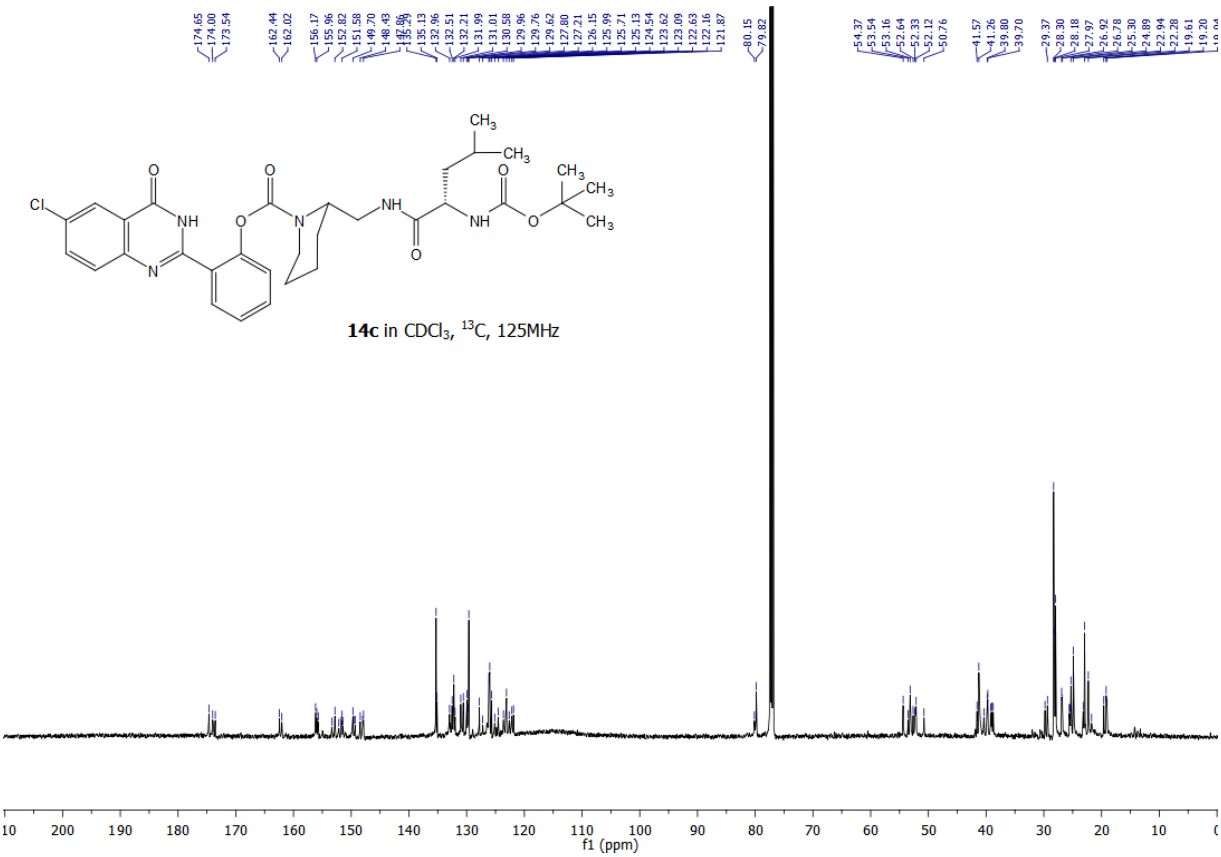
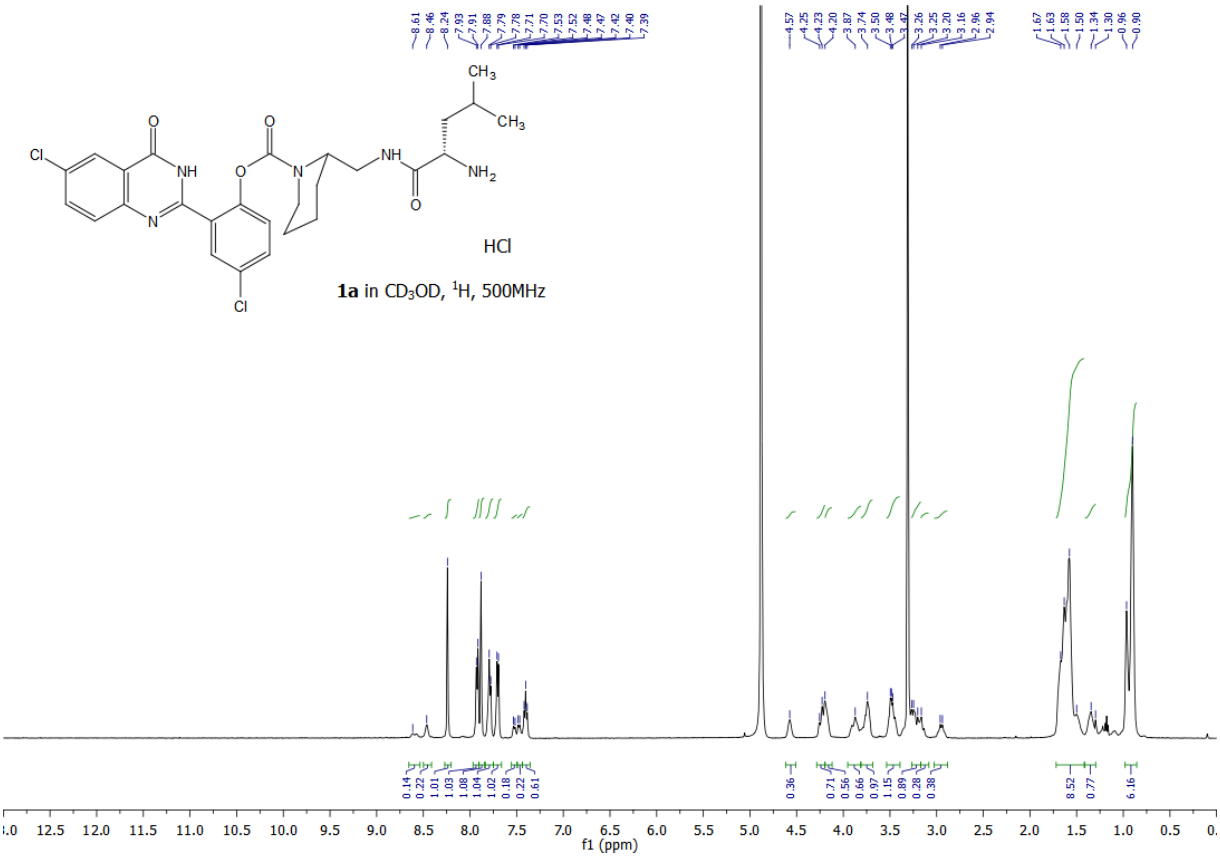
f1 (ppm)

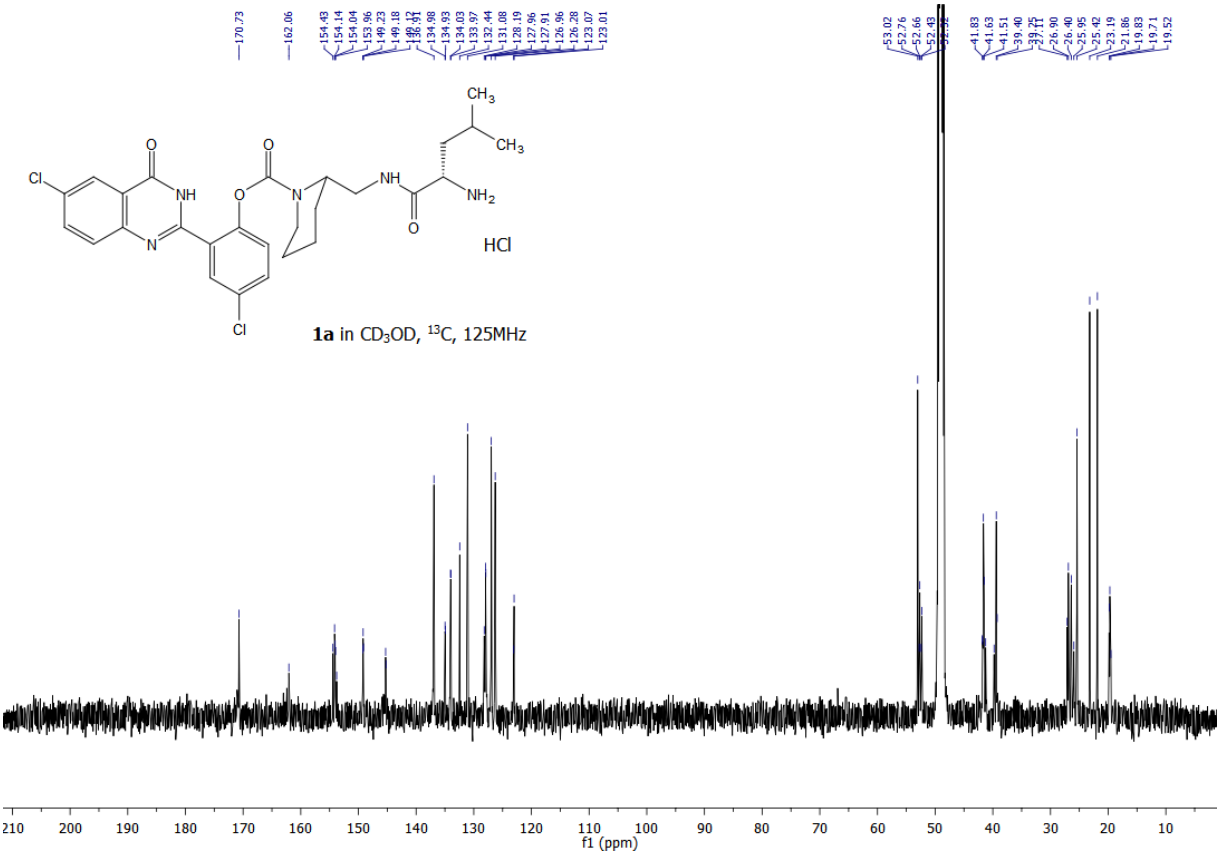

14b in CDCl3, 1H, 500MHz
f1 (ppm)

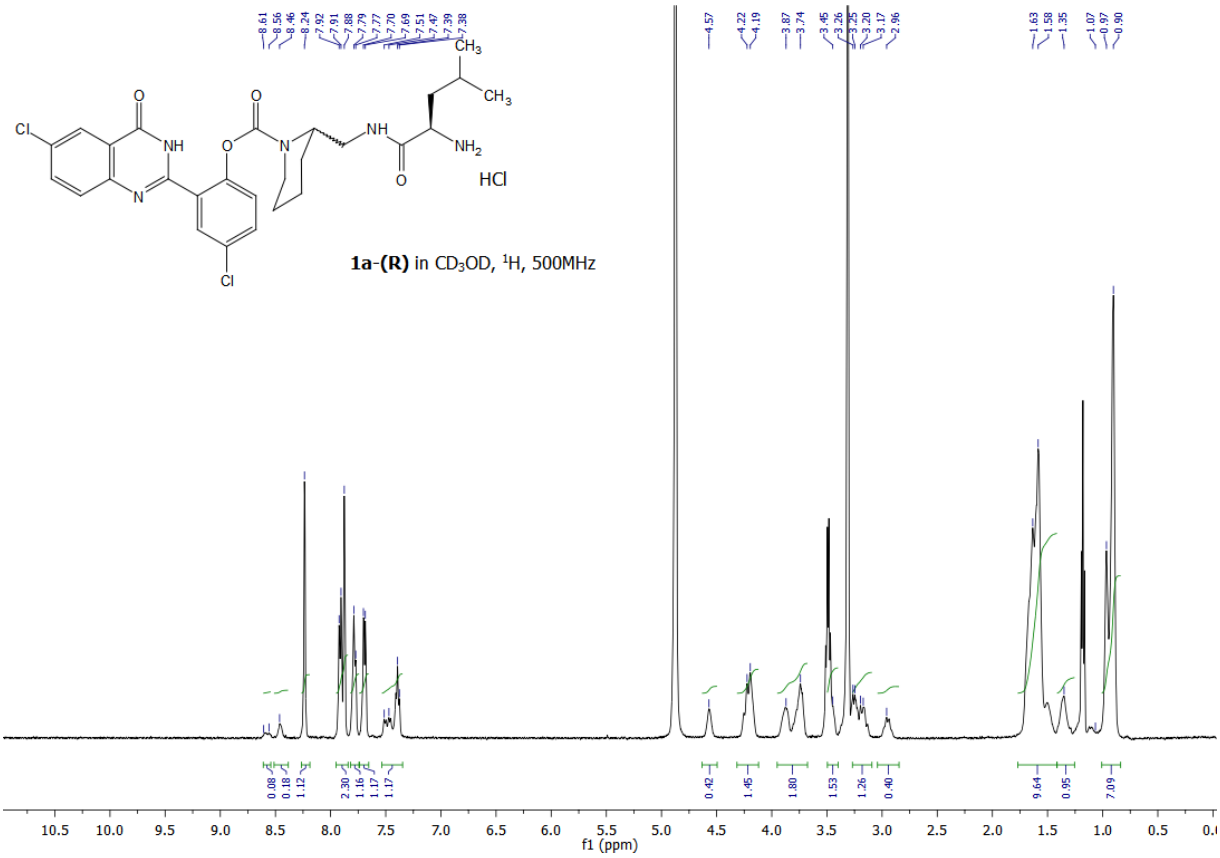

14b in CDCl3, 13C, 125MHz
f1 (ppm)

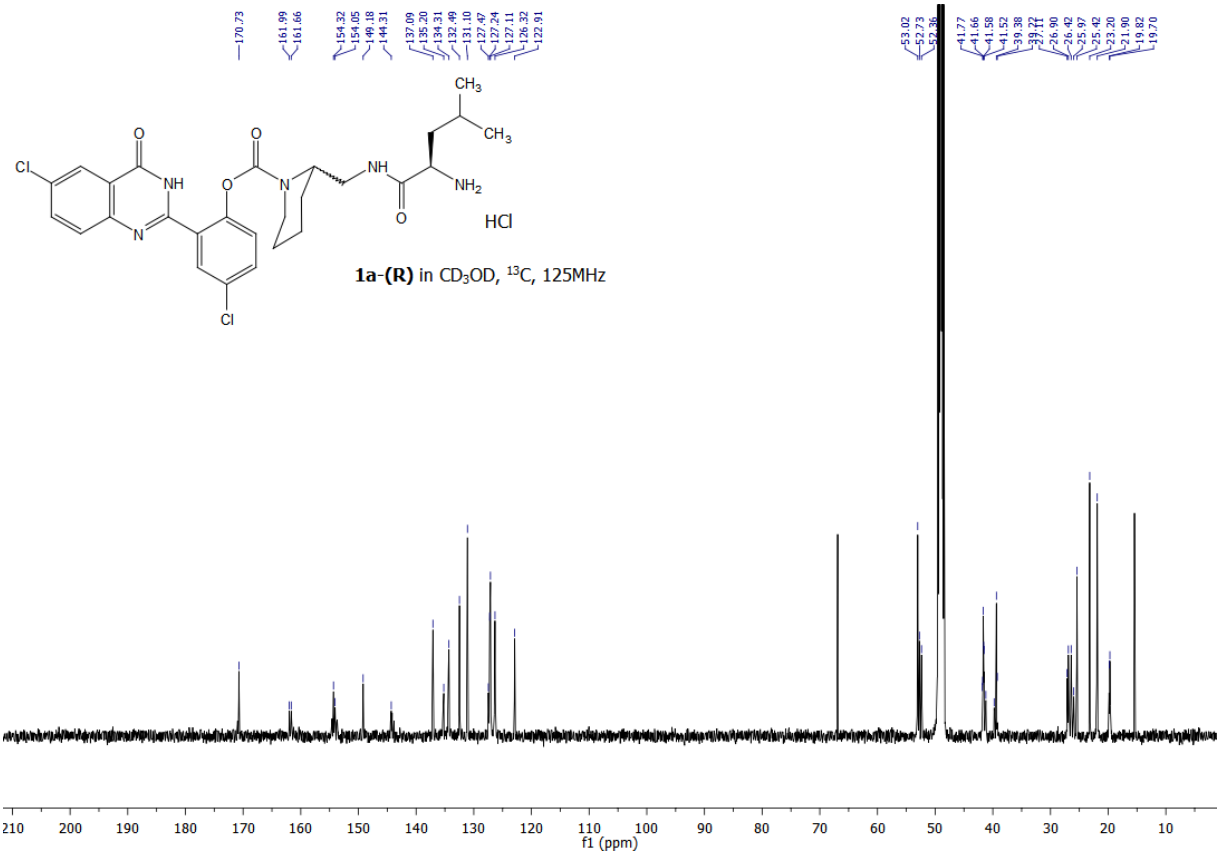

14c in CDCl3, 1H, 500MHz
f1 (ppm)


14c in CDCl3, 13C, 125MHz
f1 (ppm)

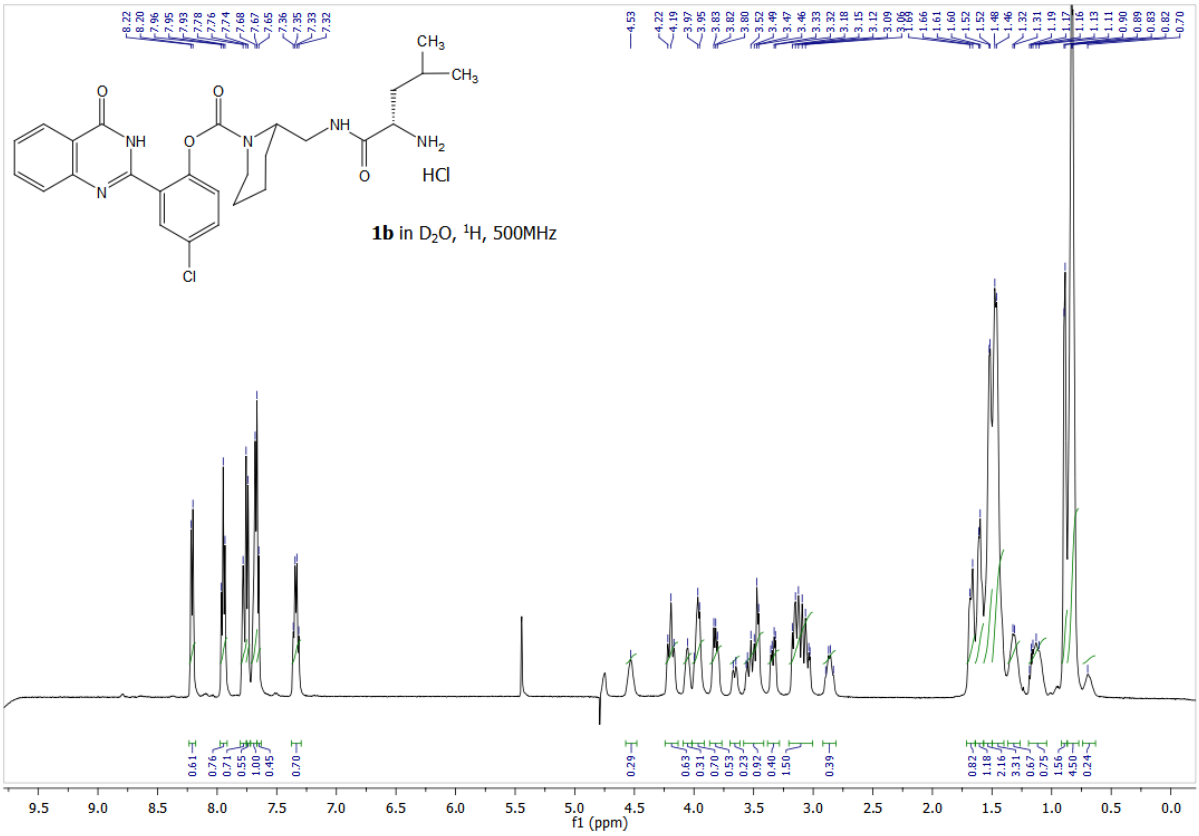

HCl
1a in CD3OD, 1H, 500MHz
f1 (ppm)

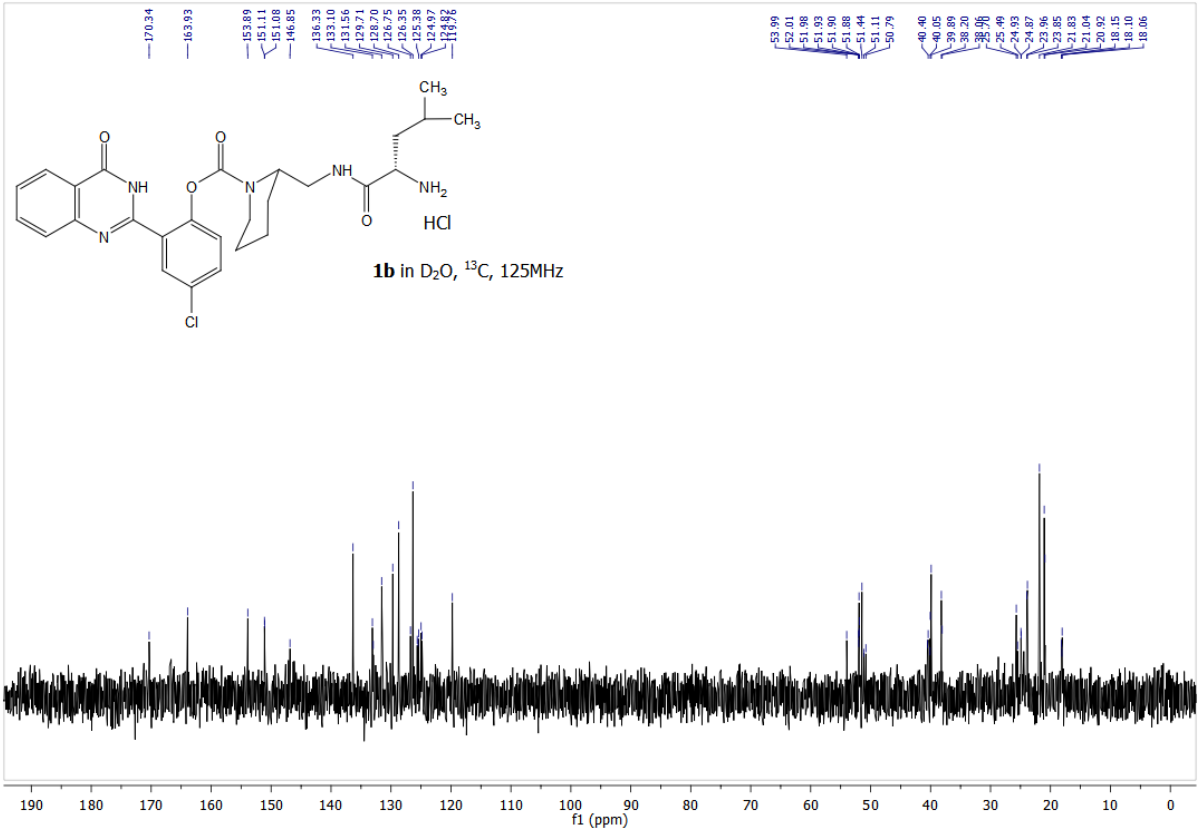

HCl
1a in CD3OD, 13C, 125MHz
f1 (ppm)

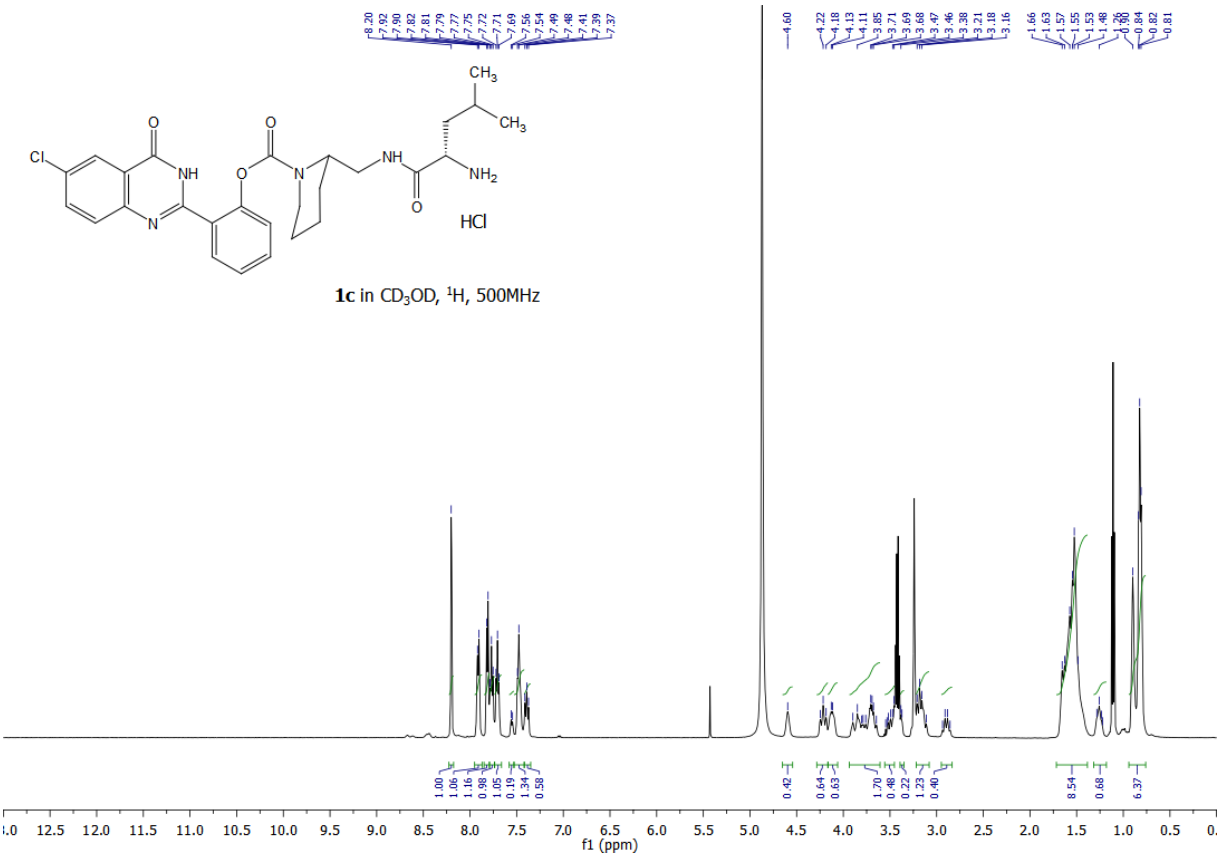

HCl
1a-(R) in CD3OD, 1H, 500MHz
f1 (ppm)

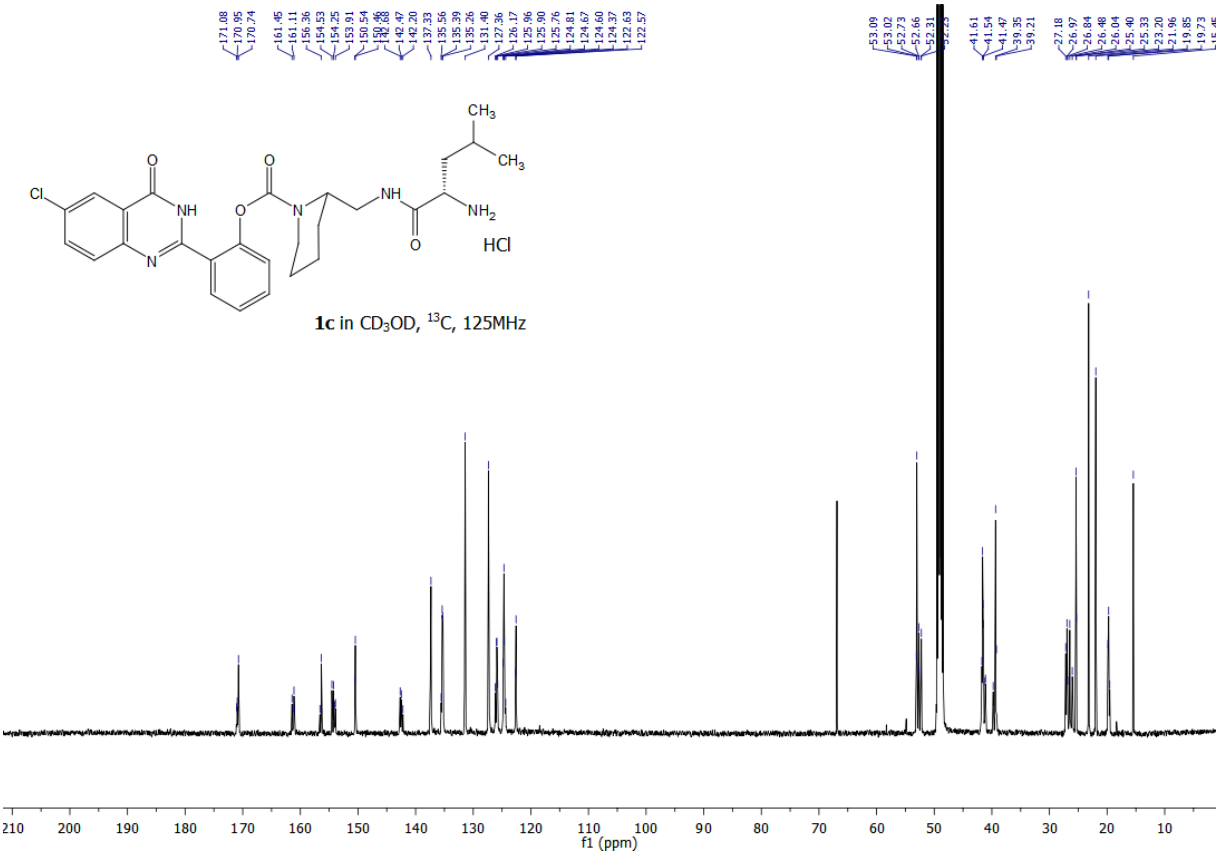

HCl
1a-(R) in CD3OD, 13C, 125MHz
f1 (ppm)

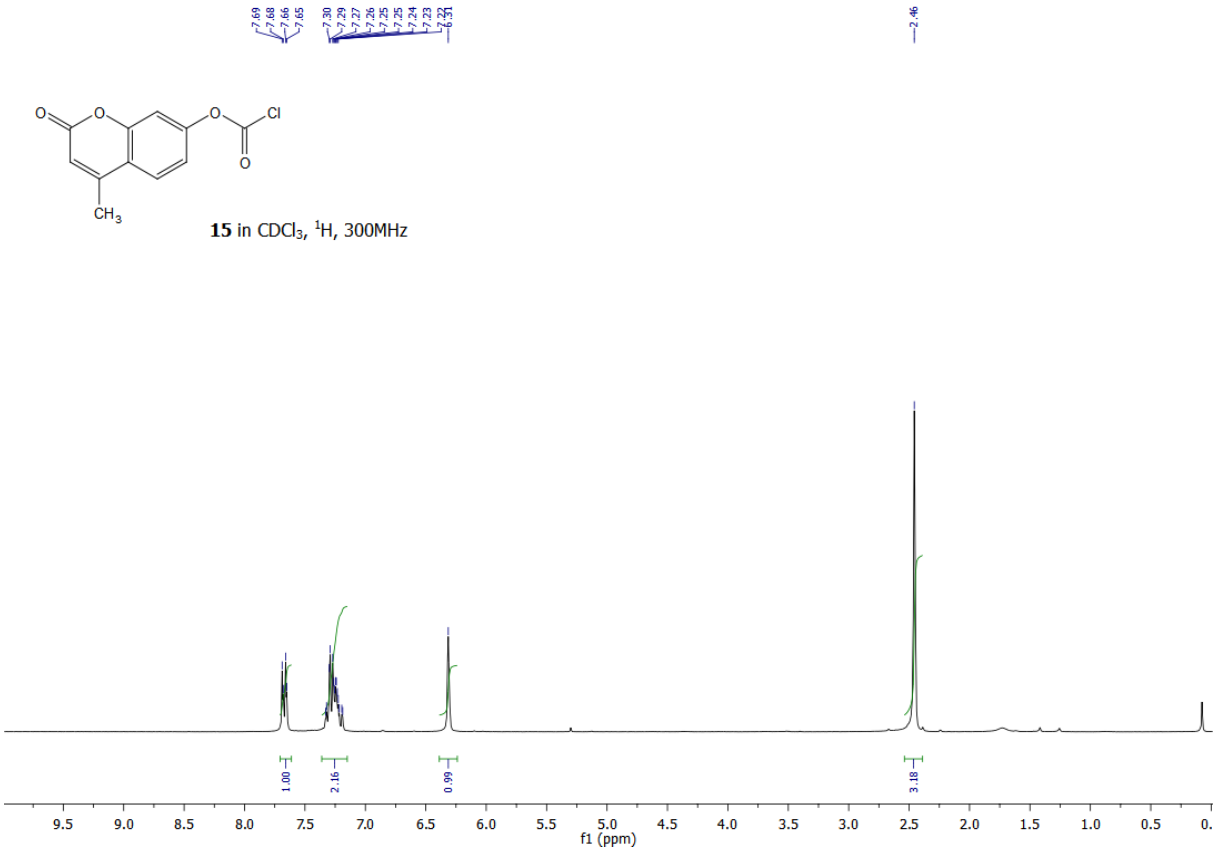
S


HCl
1b in D_2O, ^1H, 500MHz
f1 (ppm)

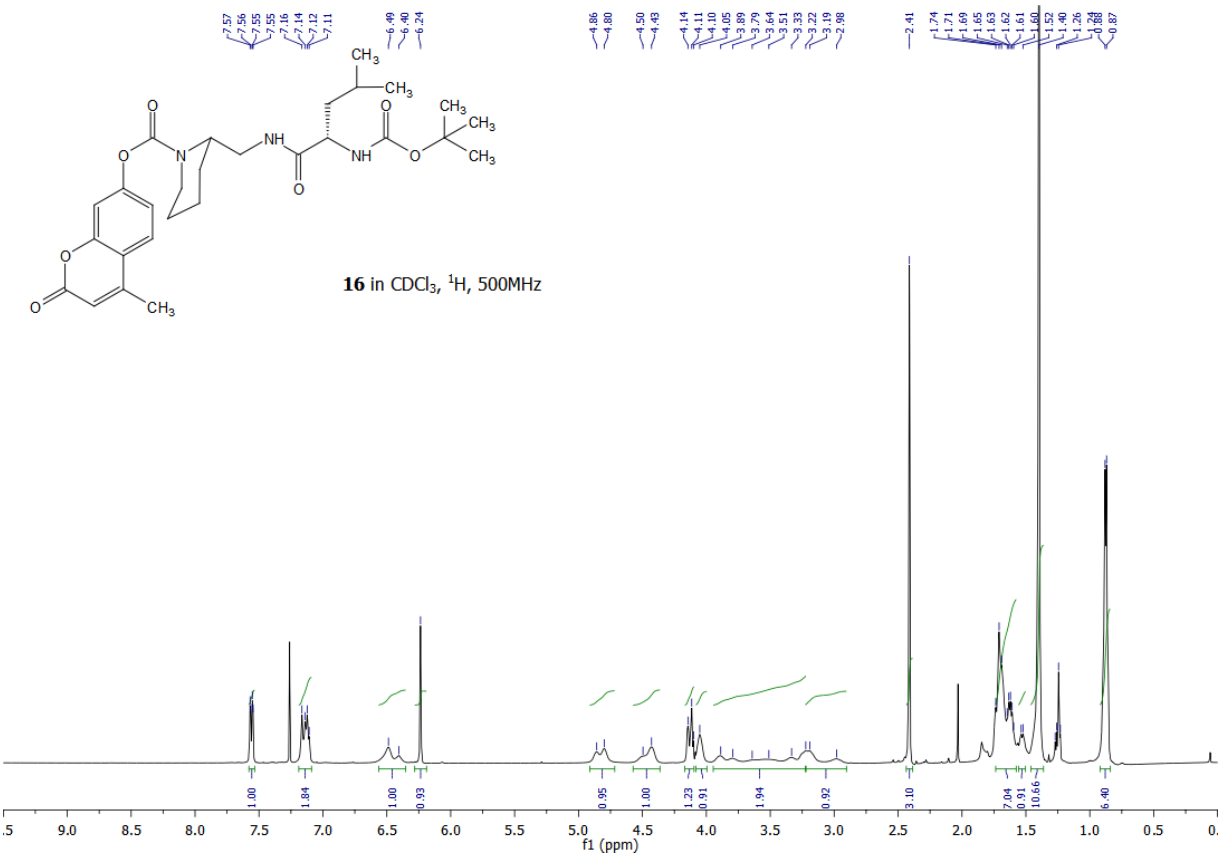

HCl
1b in D_2O, ^{13}C, 125MHz
f1 (ppm)

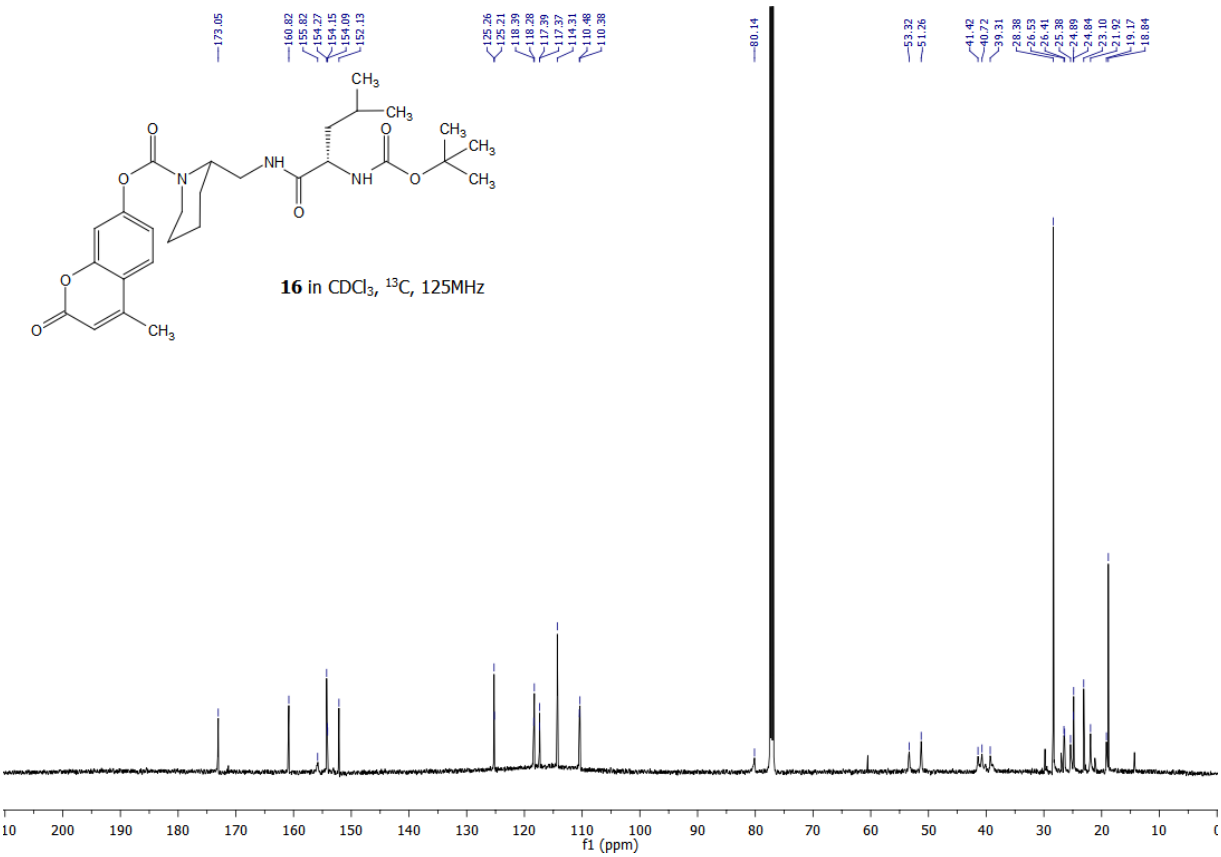

HCl
1c in CD3OD, 1H, 500MHz
f1 (ppm)

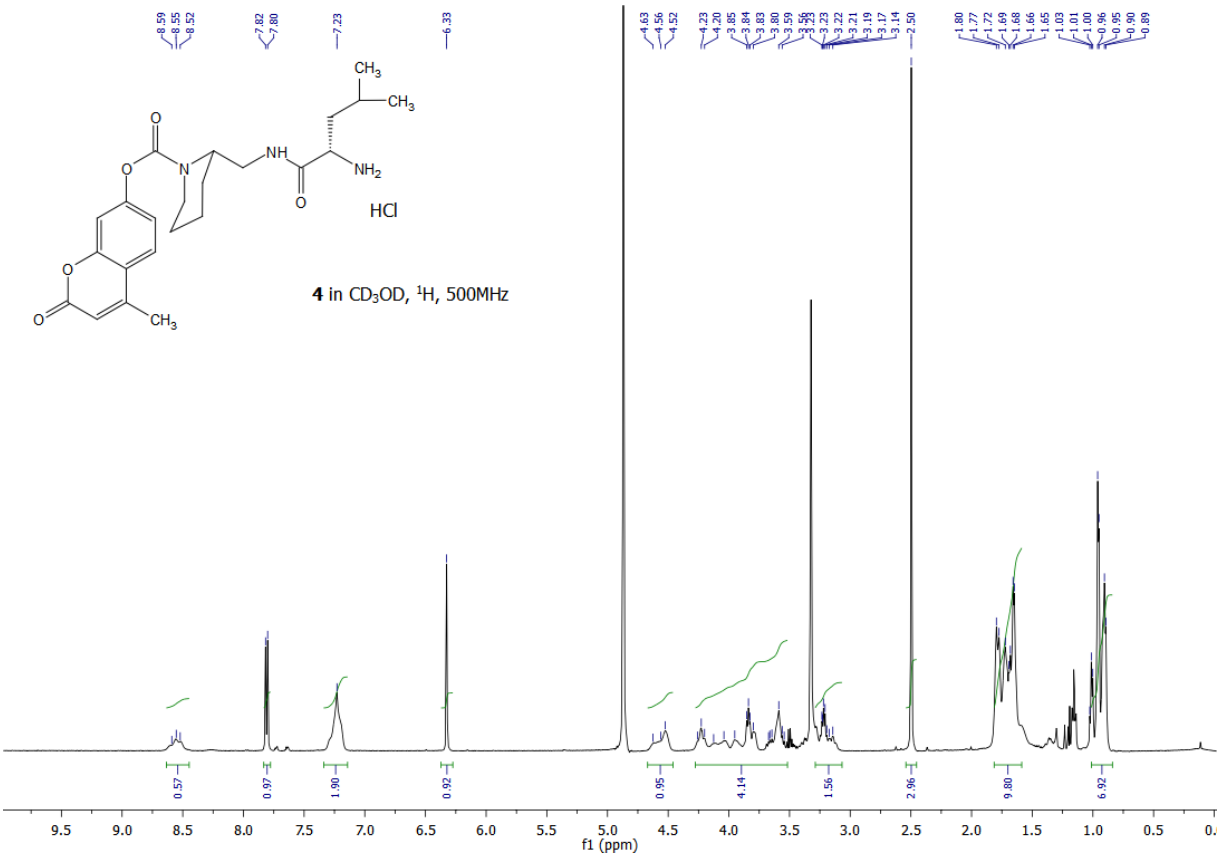

HCl
1c in CD3OD, 13C, 125MHz
f1 (ppm)

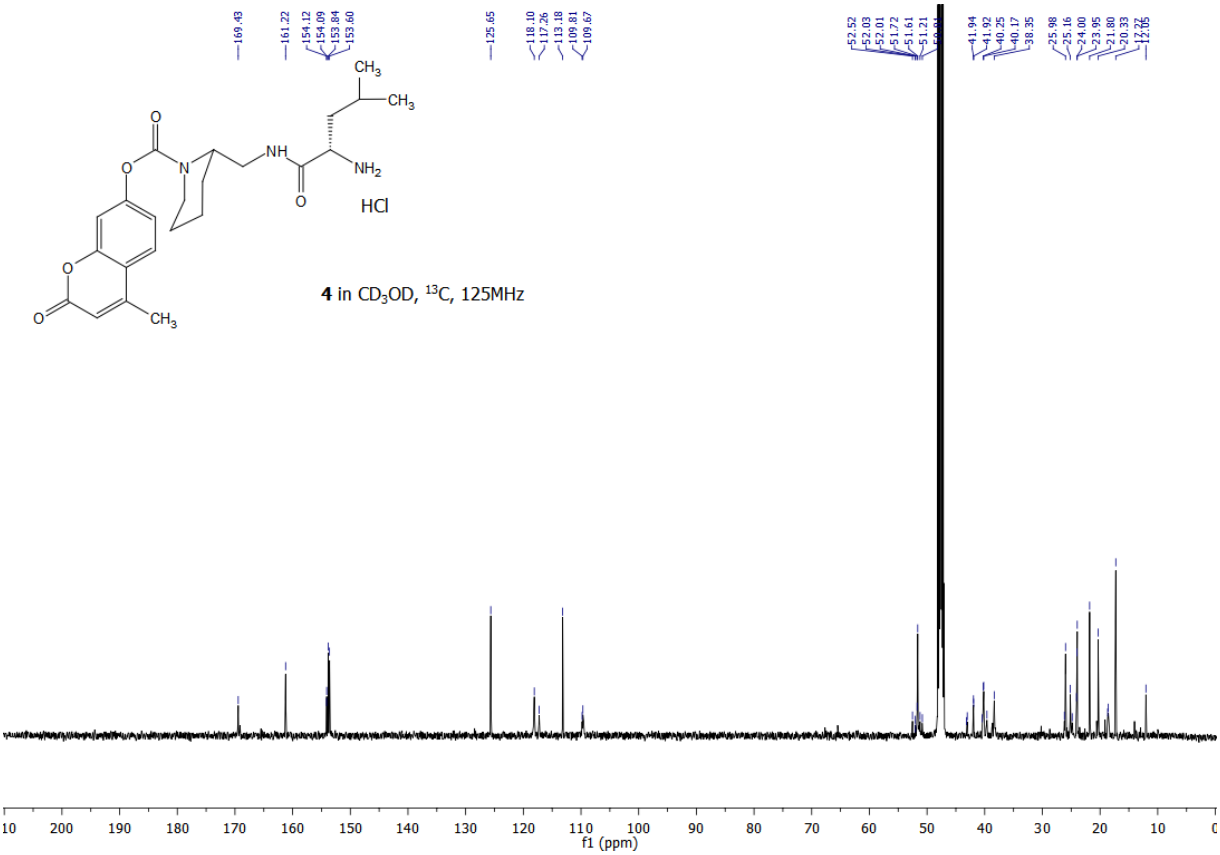


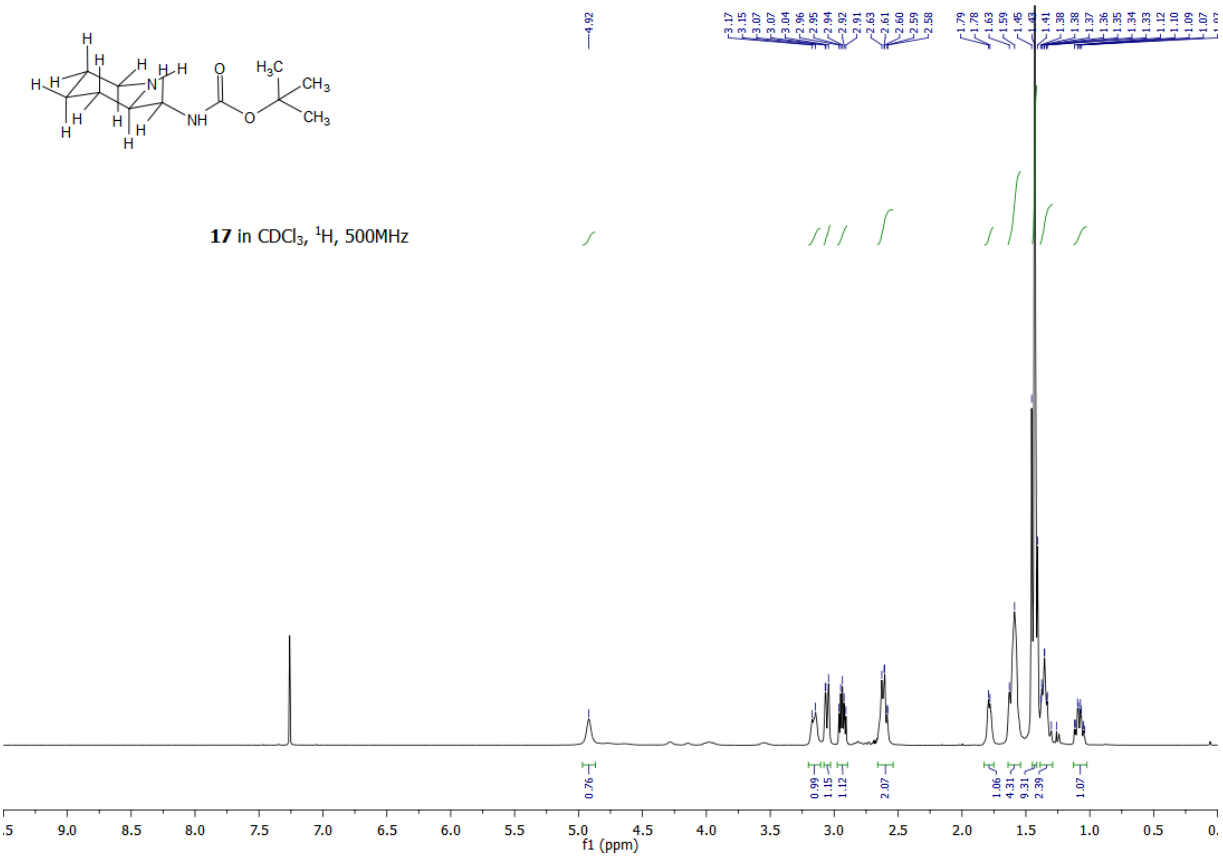
15 in $CDCl_3$, ^1H, 300MHz

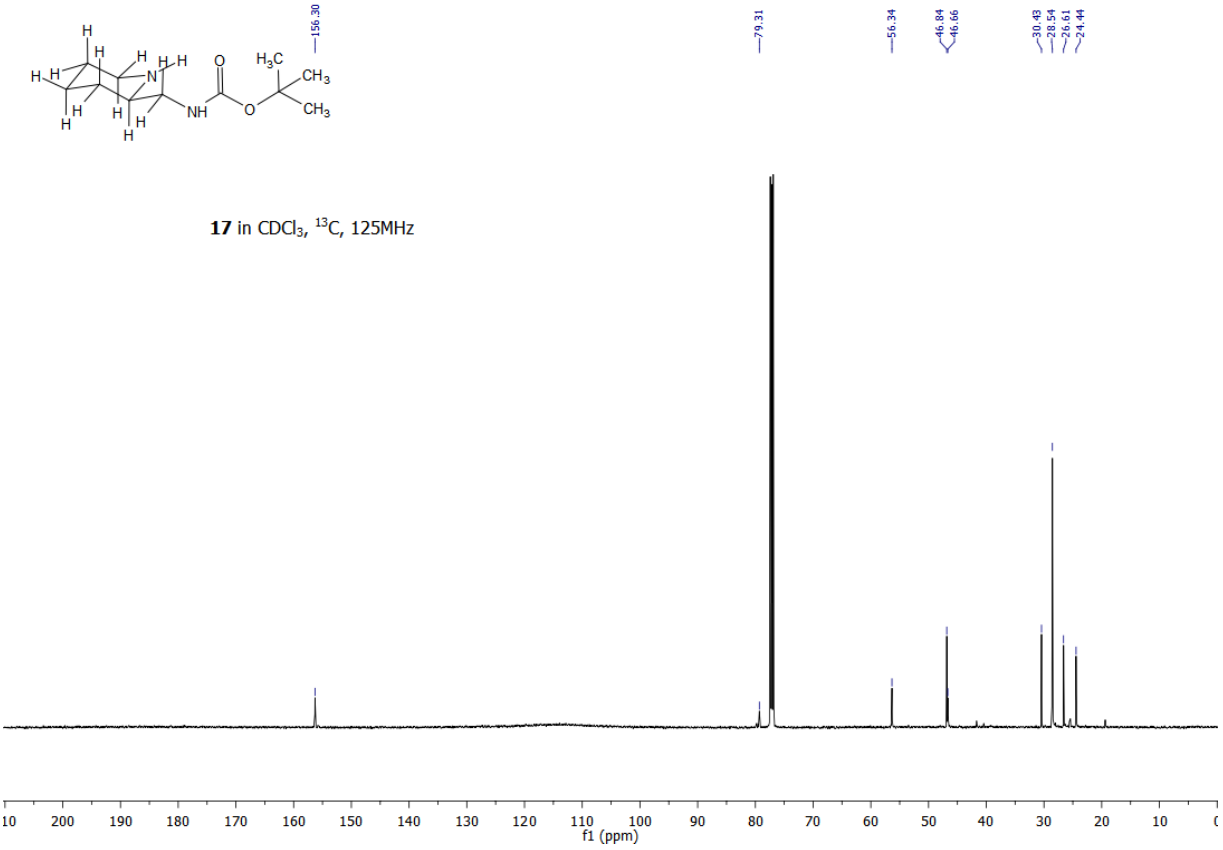

16 in CDCl3, 1H, 500MHz
f1 (ppm)

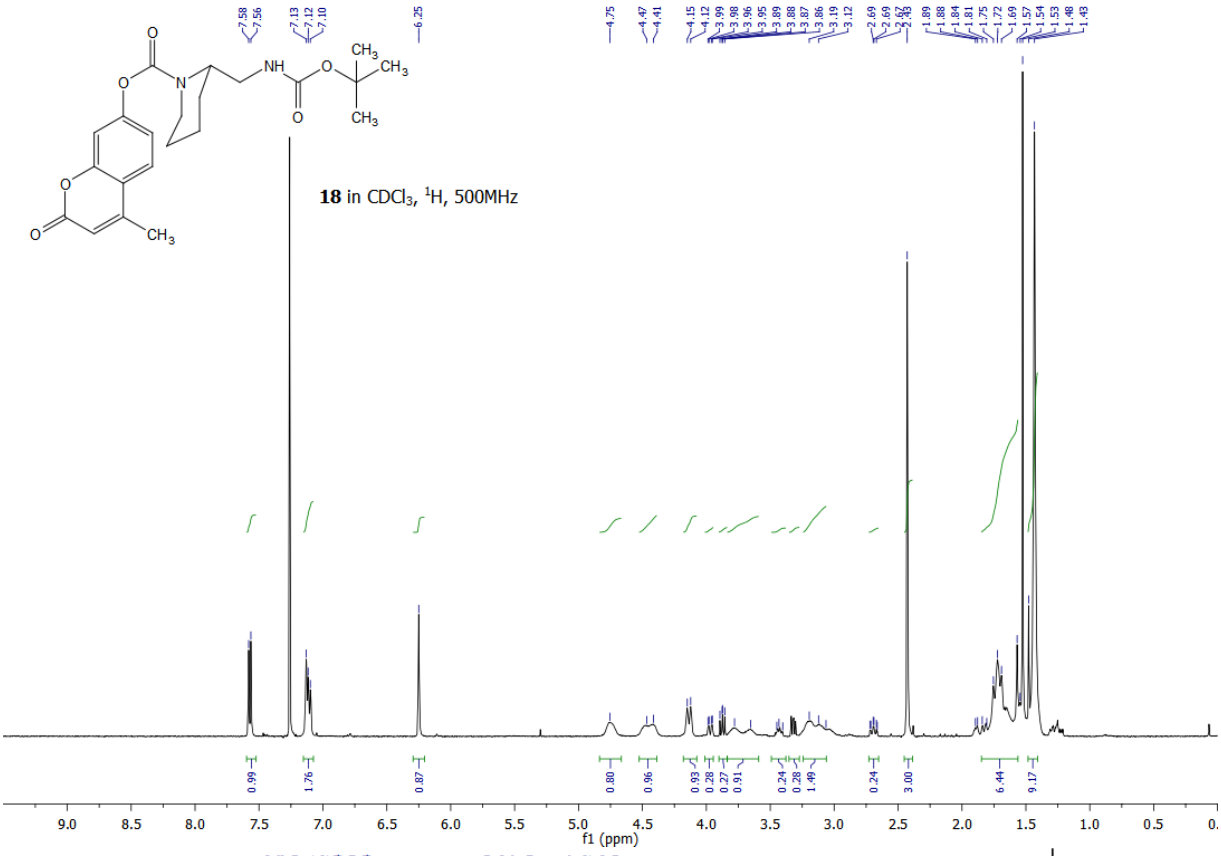

16 in CDCl3, 13C, 125MHz
f1 (ppm)

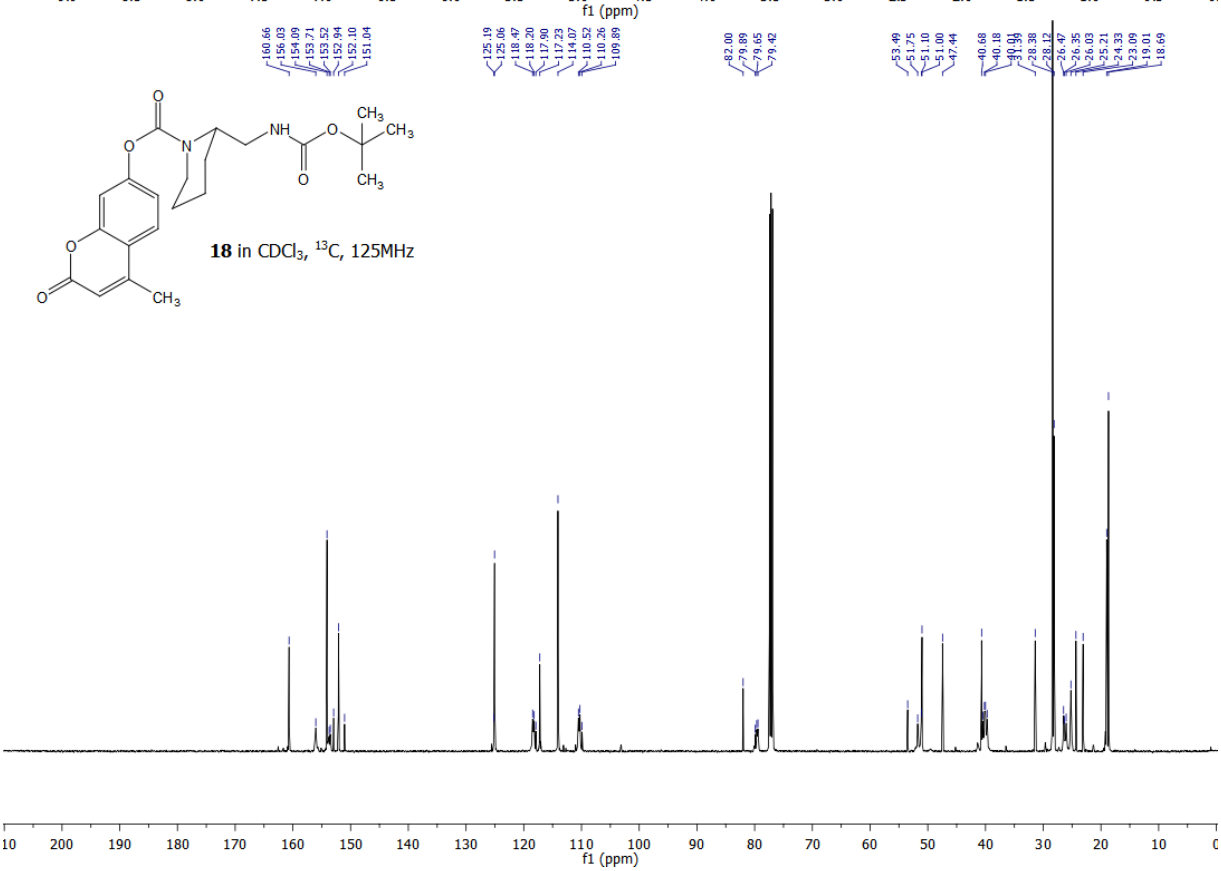

HCl
4 in CD3OD, 1H, 500MHz
f1 (ppm)

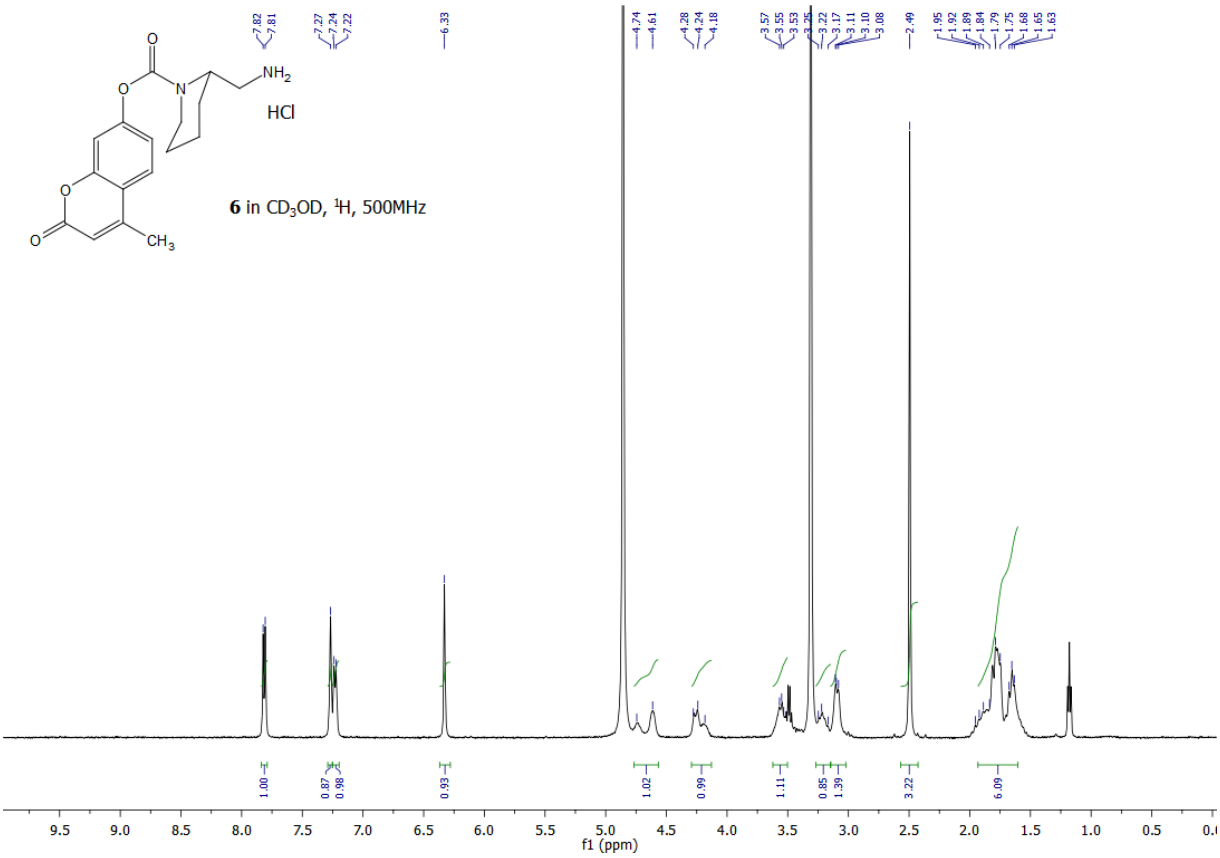

HCl
4 in CD3OD, 13C, 125MHz
f1 (ppm)

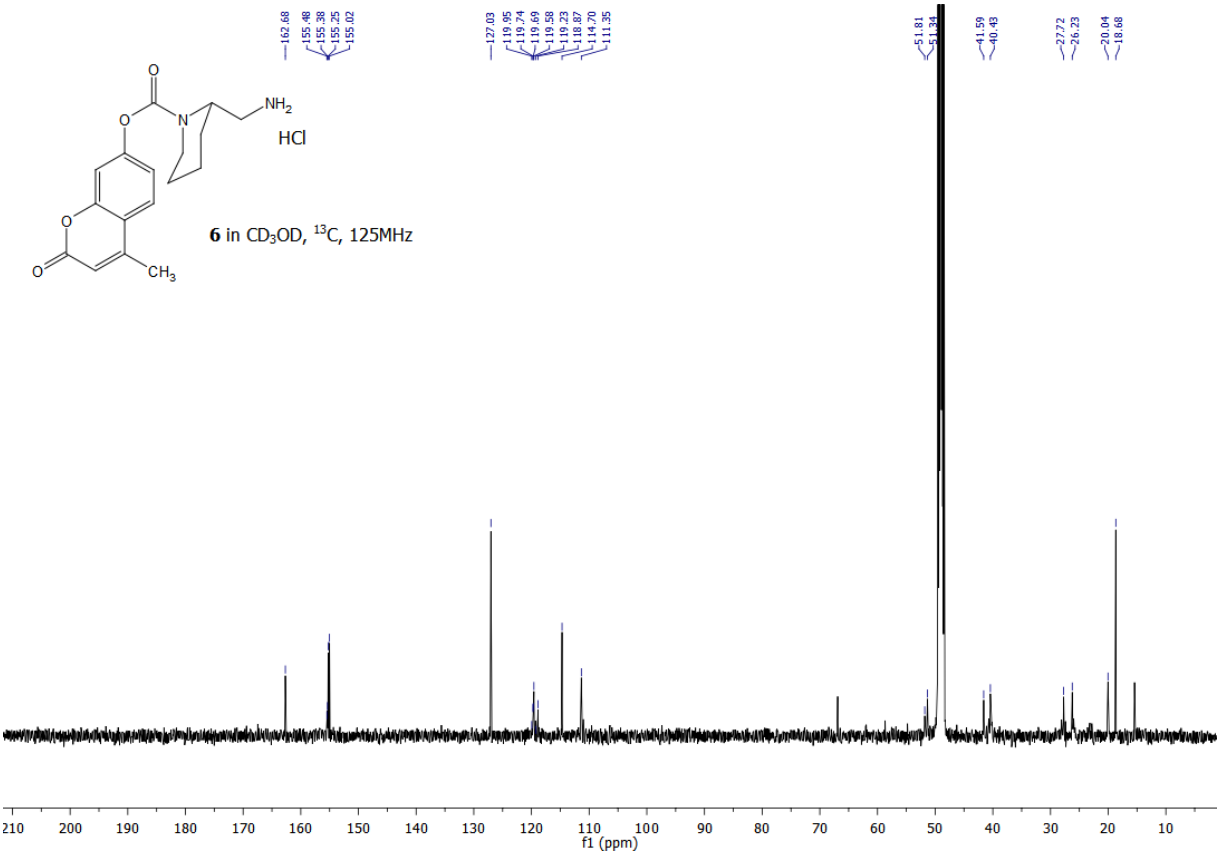

17 in CDCl3, 1H, 500MHz
f1 (ppm)


17 in CDCl3, 13C, 125MHz
f1 (ppm)


18 in CDCl3, 1H, 500MHz
f1 (ppm)


18 in CDCl3, 13C, 125MHz
f1 (ppm)


NH2
HCl
O
N
O
O
CH3
6 in CD3OD, 1H, 500MHz
7.82
7.81
7.27
7.24
7.22
6.33
4.74
4.61
4.28
4.24
4.18
3.57
3.55
3.53
3.36
3.22
3.17
3.11
3.10
3.08
2.49
1.95
1.92
1.89
1.84
1.79
1.75
1.68
1.65
1.63
1.00
0.87
0.98
0.93
1.02
0.99
1.11
0.85
1.39
3.22
6.09
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5
f1 (ppm)


NH2
HCl
O
N
O
O
CH3
6 in CD3OD, 13C, 125MHz
162.68
155.48
155.38
155.25
155.02
127.03
119.95
119.74
119.69
119.58
119.23
118.87
114.70
111.35
51.81
51.34
41.59
40.43
27.72
26.23
20.04
18.68
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10
f1 (ppm)

References

[1] O. Thorn-Seshold, M. Vargas-Sanchez, S. McKeon, J. Hasserodt, *Chem. Commun.* 2012, *48*, 6253–6255.

[2] J. Aw, Q. Shao, Y. Yang, T. Jiang, C. Ang, B. Xing, *Chem. Asian J.* 2010, *5*, 1317–1321.

[3] M. Baghbanzadeh, M. Dabiri, P. Salehi, *Heterocycles* 2008, *75*, 2809–2815.