Electronic Supplementary Material (ESI) for ChemComm.

„Double gating“ – a concept for enzyme-responsive imaging probes aiming at high tissue specificity

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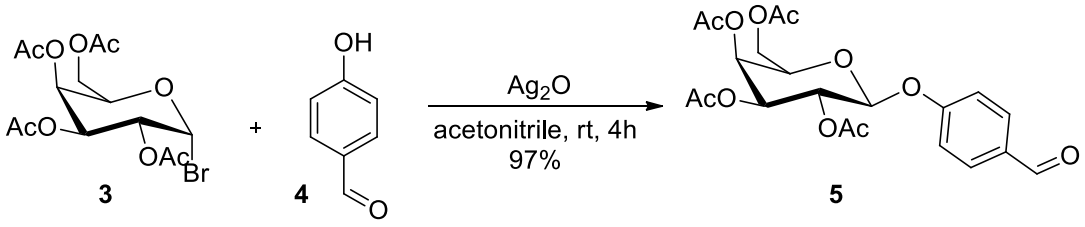
1 Chemistry

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. 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.

1.2 Synthetic procedures



5: 4-O-(2,3,4,6-Tetra-O-acetyl-β-D-galactopyranosyl)-4-oxybenzaldehyde

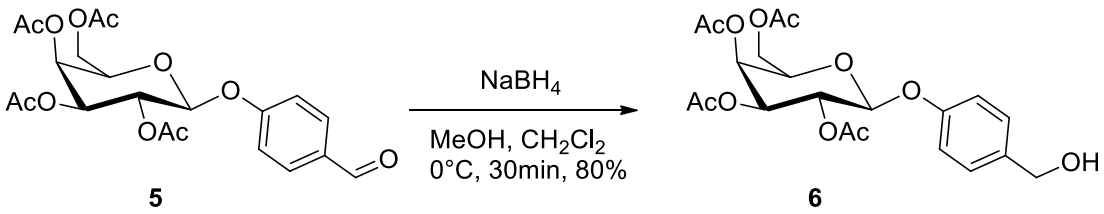
A suspension of compound **3** (1.0g, 2.31mmol, 1,0eq.), compound **4** (285mg, 2.31mmol, 1.0eq.) and silver oxide (541mg, 2.31mmol, 1.0eq.) in acetonitrile (40mL) was stirred at RT for 4h. The progress of the reaction can be monitored by TLC (cyclohexane : ethyl acetate / 6:4 / v:v). After the completion of the reaction, the volatiles were removed under reduced pressure. The crude mixture was then passed through a small plug of silicagel using cyclohexane : ethyl acetate / 1:1 / v:v as eluent. The solvent was evaporated to give **5** as colorless oil which crystallizes upon standing (1.016g, 2.25mmol, yield: 97%).

NMR: ^{1}H-NMR (300 MHz, $CDCl_3$): δ= 9.86 (s, 1H), 7.80 (d, J = 8.7 Hz, 2H), 7.07 (d, J = 8.7 Hz, 2H), 5.53 – 5.39 (m, 2H), 5.17 (d, J = 7.9 Hz, 1H), 5.10 (dd, J = 10.4, 3.4 Hz, 1H), 4.23 – 4.08 (m, 3H), 2.13 (s, 3H), 2.02 – 1.99 (m, 6H), 1.96 (s, 3H) ppm.

^{13}C-NMR (75 MHz, $CDCl_3$): δ= 190.76, 170.31, 170.18, 170.05, 169.31, 161.28, 131.80, 131.73, 116.70, 98.46, 71.27, 70.63, 68.38, 66.79, 61.37, 20.67, 20.62, 20.60, 20.53 ppm.

R_f = 0.4 (cyclohexane : ethyl acetate / 6:4 / v:v)

Analysis data are consistent with those reported in the literature.[1]



5 **6**

6: [4-(hydroxymethyl)phenyl]-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranoside)

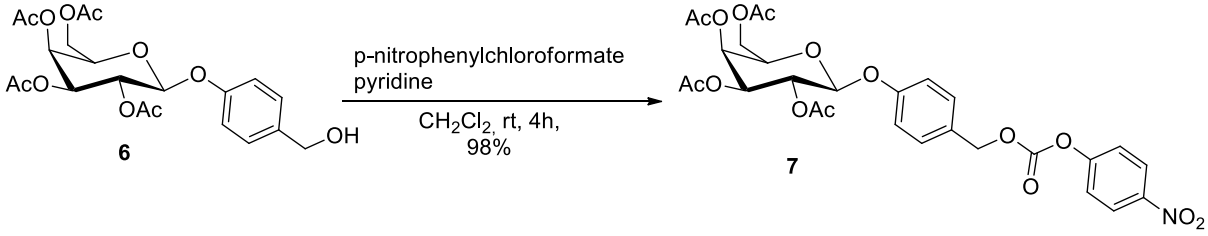
To an ice-cold solution of **5** (1.016g, 2.25mmol, 1.0eq.) in dry DCM (24mL) under an argon atmosphere was added dropwise a solution of sodium borohydride (94mg, 2.47mmol, 1.1eq.), and the reaction mixture was stirred at 0°C for 30min. The progress of the reaction can be monitored by TLC (cyclohexane : ethyl acetate / 6:4 / v:v). After the completion of the reaction, it was quenched with NH_4Cl sat. (50mL), the organic layer was separated and the aqueous layer was washed three times with DCM (3x50mL). The combined organic layers were dried with Na_2SO_4, filtered and evaporated to dryness. The crude mixture was then purified via column chromatography on silicagel (cyclohexane : ethyl acetate / 6:4 / v:v) to obtain **6** as white solid (815mg, 1.80mmol, yield: 80%).

NMR: ^{1}H-NMR (300 MHz, $CDCl_3$): δ= 7.31 (d, *J* = 8.6 Hz, 2H), 6.99 (d, *J* = 8.6 Hz, 2H), 5.53 – 5.43 (m, 2H), 5.11 (dd, *J* = 10.4, 3.4 Hz, 1H), 5.03 (d, *J* = 7.9 Hz, 1H), 4.65 (d, *J* = 3.0 Hz, 2H), 4.28 – 4.01 (m, 3H), 2.18 (s, 3H), 2.07+2.06 (2xs, 6H), 2.01 (s, 3H) ppm.

^{13}C-NMR (75 MHz, $CDCl_3$): δ= 170.38, 170.27, 170.16, 135.90, 128.50, 117.05, 99.77, 71.04, 70.83, 68.63, 66.86, 64.81, 61.36, 20.68, 20.61 ppm.

R_f = 0.30 (cyclohexane : ethyl acetate / 6:4 / v:v)

Analysis data are consistent with those reported in the literature.[1]



6 **7**

7: [4-(4-nitro-phenoxycarbonyloxymethyl)phenyl]-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranoside)

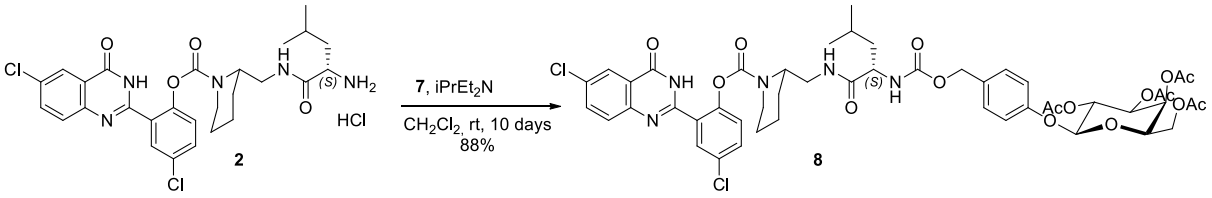
To a solution of **6** (230mg, 0.50mmol, 1.0eq.) in dry DCM (10mL) was successively added paranitrophenylchloroformate (231mg, 1.11mmol, 2.2eq.) and pyridine (105µL, 1.27mmol, 2.5eq.) at RT. The reaction mixture was stirred at this temperature during 5h. The progress of the reaction can be monitored by TLC (cyclohexane : ethyl acetate / 6:4 / v:v). After the completion of the reaction, it was quenched with water (20mL), the organic layer was separated and the aqueous layer was washed once with DCM (20mL). The combined organic layers were dried with Na_2SO_4, filtered and evaporated to dryness. The crude mixture was then purified via column chromatography on silicagel (cyclohexane : ethyl acetate / 65:35 / v:v) to obtain **7** as white solid (303mg, 0.49mmol, yield: 98%).

NMR: ^{1}H-NMR (300 MHz, $CDCl_3$): δ= 8.27 (d, *J* = 9.0 Hz, 2H), 7.38 (t, *J* = 8.4 Hz, 4H), 7.03 (d, *J* = 8.4 Hz, 2H), 5.54 – 5.43 (m, *J* = 10.1, 7.9 Hz, 2H), 5.24 (s, 2H), 5.12 (dd, *J* = 10.5, 3.3 Hz, 1H), 5.07 (d, *J* = 7.9 Hz, 1H), 4.28 – 4.04 (m, 3H), 2.18 (s, 3H), 2.06 (s, 6H), 2.02 (s, 3H) ppm.

^{13}C-NMR (75 MHz, $CDCl_3$): δ= 170.46, 170.33, 170.23, 169.47, 157.57, 155.63, 152.56, 145.57, 130.67, 129.23, 125.44, 121.88, 117.22, 99.56, 71.26, 70.90, 70.62, 68.73, 66.96, 61.46, 20.85, 20.78, 20.70 ppm.

R_f = 0.18 (cyclohexane : ethyl acetate / 7:3 / v:v)

Analysis data are consistent with those reported in the literature.[1]



8: (2R,3S,4S,5R,6S)-2-(acetoxymethyl)-6-(4-(((((S)-1-((((S)-1-((4-chloro-2-(6-chloro-4-oxo-3,4-dihydroquinazolin-2-yl)phenoxy)carbonyl)piperidin-2-yl)methyl)amino)-4-methyl-1-oxopentan-2-yl)carbamoyl)oxy)methyl)phenoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate

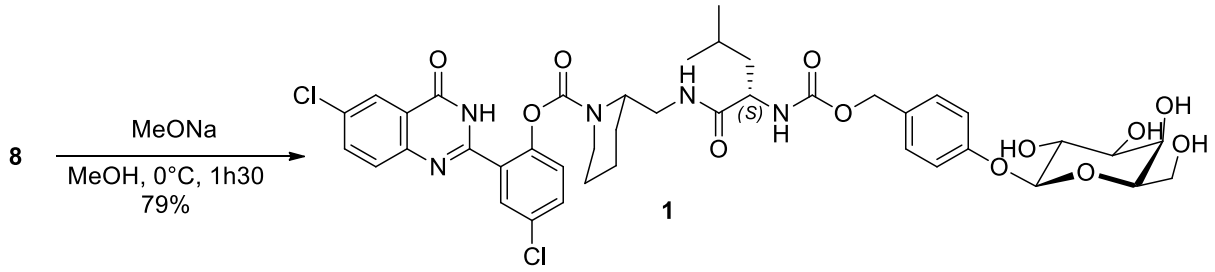
To a solution of **2**[2] (40mg, 0.067mmol, 1.0eq.) and DIPEA (30µL, 0.168mmol, 2.5eq.) in dry DCM (5mL) under an argon atmosphere wad added **7** (42mg, 0.067mmol, 1.0eq.) at RT. The reaction mixture was stirred at this temperature during 10 days. The progress of the reaction can be monitored by TLC (petroleum ether : ethyl acetate / 4:6 / v:v). After the completion of the reaction, it was diluted with DCM (10mL), and washed twice with water (2x20mL). The organic layer was dried with Na_2SO_4, filtered and evaporated to dryness. The crude mixture was then purified via column chromatography on silicagel (gradient of petroleum ether : ethyl acetate / 6:4, 5:5, 4:6 / v:v) to obtain **8** as white solid (61mg, 0.059mmol, yield: 88%).

NMR: ^{1}H-NMR (500 MHz, $CDCl_3$): δ= 12.09 – 11.20 (m, 0.6H), 8.51 (s, 0.5H), 8.29 (d, *J* = 11.9 Hz, 0.5H), 8.21 (m, 0.3H), 8.11 – 7.96 (m, 0.7H), 7.90 (d, *J* = 9.1 Hz, .05H), 7.84 – 7.65 (m, 2H), 7.50 – 7.34 (m, 0.7H), 7.33 – 7.11 (m, 1.5H), 7.07 (d, *J* = 8.3 Hz, 0.5H), 7.02 – 6.76 (m, 3H), 5.48 – 5.36 (m, 2H), 5.32 (d, *J* = 8.1 Hz, 0.4H), 5.28 (s, 2H), 5.13 – 5.04 (m, 1H), 5.03 – 4.92 (m, 1H), 4.85 – 4.65 (m, 70.4), 4.45 – 4.26 (m, 1.5H), 4.26 – 3.97 (m, 5H), 3.88 – 3.08 (m, 2H), 2.87 – 2.66 (m, 0.7H), 2.16 (s, 3H), 2.04 (s, 6H), 1.99 (s, 3H), 1.88 (M, 0.4H), 1.76 – 1.31 (m, 9H), 1.01 – 0.79 (m, 7H) ppm.

^{13}C-NMR (125 MHz, $CDCl_3$): δ= 174.32, 173.38, 173.31, 173.07, 170.43, 170.34, 170.22, 169.47, 162.52, 162.26, 162.13, 156.77, 156.72, 156.61, 156.52, 154.32, 152.71, 152.29, 151.49, 150.58, 150.34, 148.81, 148.39, 148.13, 147.97, 147.69, 135.62, 135.57, 135.49, 135.36, 133.44, 133.27, 132.96, 132.24, 131.72, 131.35, 131.24, 131.08, 130.81, 130.63, 130.35, 130.04, 129.76, 129.70, 129.61, 129.23, 128.51, 127.93, 126.14, 126.04, 126.00, 125.75, 125.38, 124.74, 124.17, 123.35, 122.00, 121.85, 117.08, 116.99, 116.83, 116.80, 99.69, 99.65, 71.11, 71.07, 70.89, 68.69, 66.94, 66.70, 65.96, 61.46, 61.41, 53.58, 53.54, 53.27, 52.83, 50.32, 40.99, 40.81, 40.72, 40.47, 40.41, 39.57, 39.00, 38.33, 29.76, 27.37, 26.99, 25.56, 24.81, 23.95, 23.61, 23.07, 22.77, 22.65, 22.61, 22.38, 22.30, 20.91, 20.81, 20.74, 20.66, 19.25, 19.15, 17.58, 17.37, 14.74 ppm.

HRMS: $C_{49}H_{56}Cl_2N_5O_{16}$ $[M+H]^+$ m/z found: 1040.3045 calc. 1040.3094

R_f = 0.45 (petroleum ether : ethyl acetate / 4:6 / v:v)



1: (S)-4-chloro-2-(6-chloro-4-oxo-3,4-dihydroquinazolin-2-yl)phenyl 2-(((S)-4-methyl-2-((((4-(((2S,3R,4S,5R,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)benzyl)oxy)carbonyl)amino)pentanamido)methyl)piperidine-1-carboxylate

To an ice-cold solution of **8** (61mg, 0.059mmol, 1.0eq.) in dry MeOH (6mL) under an argon atmosphere wad added sodium (14mg, 0.59mmol, 10eq.) in dry MeOH (4mL). The reaction mixture was stirred at this temperature during 1h30. The progress of the reaction can be monitored by MS analysis (petroleum ether : ethyl acetate / 4:6 / v:v). After the completion of the reaction, Amberjet 1000H was added to the mixture until the pH gets neutral. It was then filtered and evaporated to dryness. The crude mixture was then purified via column chromatography on silicagel (gradient of DCM : MeOH / 94:6, 93:7 / v:v) to obtain **1** as white solid (40mg, 0.046mmol, yield: 79%).

NMR: ^{1}H-NMR (500 MHz, CD_3OD): δ= 8.20 (s, 1H), 7.87 – 7.78 (m, *J* = 8.7 Hz, 2H), 7.78 – 7.69 (m, *J* = 8.0 Hz, 1H), 7.61 – 7.44 (m, *J* = 33.8 Hz, 1H), 7.42 – 7.17 (m, 3H), 7.07 (d, *J* = 8.3 Hz, 2H), 4.98 (s, 2H), 4.80 – 4.70 (m, 0.5H), 4.62 – 4.49 (m, 0.5H), 4.28 – 3.99 (m, *J* = 44.4 Hz, 2H), 3.92 (s, 1H), 3.86 – 3.71 (m, 4H), 3.71 – 3.54 (m, *J* = 47.0 Hz, 2.5H), 3.54 – 3.42 (m, *J* = 10.6 Hz, 0.5H), 3.42 – 3.30 (m, *J* = 19.4 Hz, 2.5H), 3.26 – 2.86 (m, 2H), 1.74 – 1.27 (m, 9H), 0.89 (s, 6H) ppm.

^{13}C-NMR (125 MHz, CD_3OD): δ= 175.74, 175.51, 163.12, 159.02, 158.38, 154.43, 154.24, 154.08, 152.57, 150.81, 149.19, 148.58, 138.17, 136.24, 134.03, 132.87, 132.06, 131.87, 131.77, 130.98, 130.81, 130.53, 130.42, 130.12, 129.84, 126.47, 125.05, 123.42, 117.66, 102.85, 76.91, 74.81, 72.25, 70.17, 67.35, 62.37, 55.08, 53.47, 53.00, 52.52, 52.32, 42.15, 42.00, 41.59, 41.39, 41.03, 39.50, 39.22, 39.07, 33.05, 30.75, 28.44, 27.32, 27.03, 26.88, 26.35, 26.07, 25.86, 23.89, 23.45, 21.88, 21.74, 19.83 ppm.

HRMS: $C_{41}H_{48}Cl_2N_5O_{12}$ $[M+H]^+$ m/z found: 872.2666 calc. 872.2671

R_f = 0.42 (DCM : MeOH / 9:1 / v:v)

2 Biology

2.1 In vitro analyses

2.1.1 Both LAP and β-Gal activities are required to get fluorescence from probe 1

To a solution of probe **1** in PBS (Dulbecco's Phosphate Buffer Saline, Invitrogen Corp.) preheated to 37°C in a 96-well black plate (Microfluor®, Thermo Scientific) was added either a mixture of LAP (Leucine Aminopeptidase, microsomal from porcine kidney, Type IV-S, Sigma-Aldrich) and β-Gal (β-Galactosidase from *Escherichia coli,* Grade VIII, Sigma-Aldrich) in PBS or LAP only, or β-Gal only, or none of these enzymes.

Final concentration of probe **1**: 100µM

Final concentration of LAP: 0.007U

Final concentration of β-Gal: 1U

Final concentration of DMSO: 0.1%

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 - λ_{ex} = 360 nm, λ_{em}= 530 nm). The resulting curves (Figure 2.A and (Figure 2.B) are the mean of triplicates.

2.1.2 Influence of probe concentration on fluorescence signal

The same protocol was used as in 2.2.1 but with a range of concentrations of probe **1** incubated with the mixture of LAP and β-Gal.

Final concentration of probe **1**: range from 1µM to 10µM

Final concentration of LAP: 0.007U

Final concentration of β-Gal: 1U

Final concentration of DMSO: from 0.001% to 0.01%

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 - λ_{ex} = 360 nm, λ_{em}= 530 nm). The resulting curves (Figure 2.C) are the mean of triplicates.

2.2 In cellulo assay

2.2.1 Cell culture

HeLa and C17-2 (stably transfected with LacZ gene) 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.

2.2.2 Incubation of cells with probe 1

8.10^4 HeLa cells or C17-2 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 the medium was replaced by 450µL of supplemented DMEM. 50µL of a stock solution of probe **1** at 500 µM (in PBS, containing 1 % DMSO) to have a final concentration of 50 µM in 500 µL final volume. Cells were then incubated for 4 h before images were taken (Figure 3).

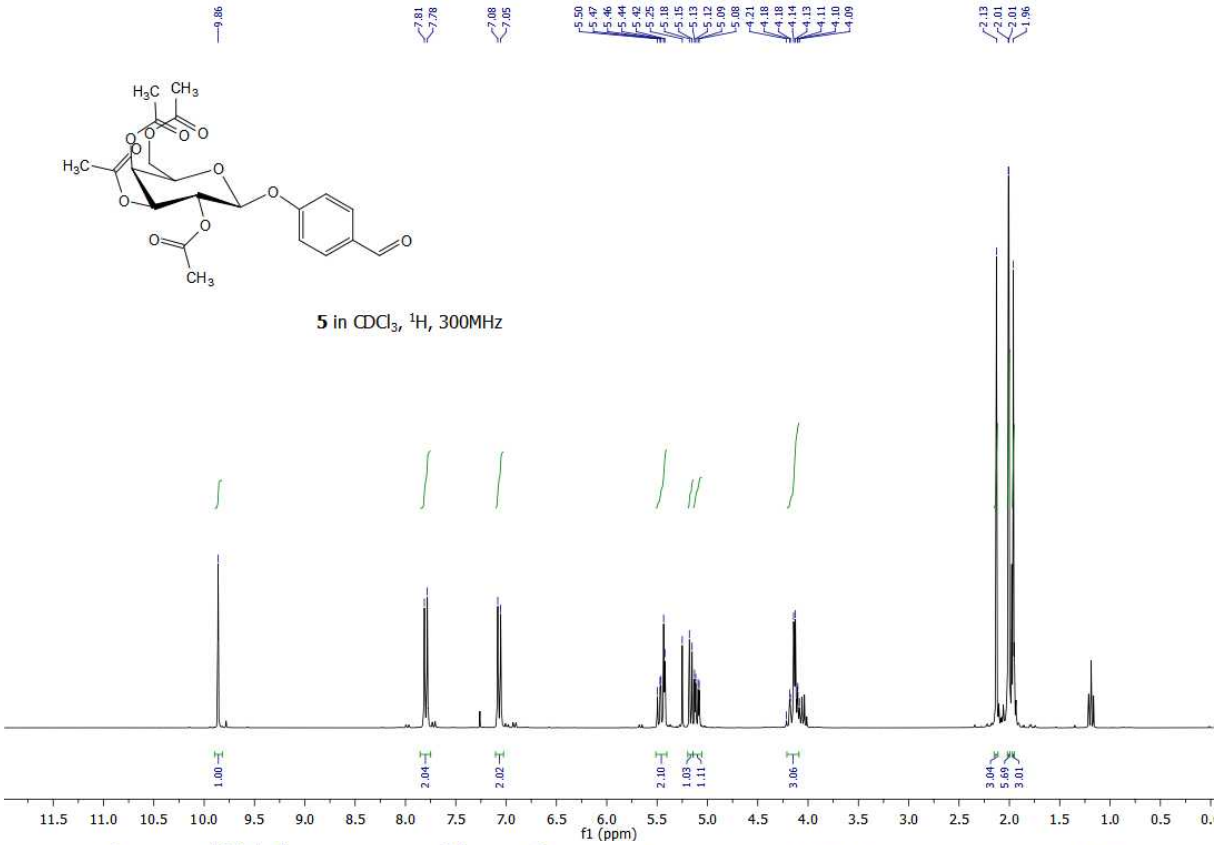
Fluorescence images were captured using a Zeiss AxioObserver Z1 instrument with EC Plan Neofluar 10x objective lens. The light source was metal halide fluorescence HPX 100. For fluorescence imaging the Zeiss filter set 21HE was used with λ_{ex}= 325 – 355 nm and λ_{em}= 470 – 555 nm. Images were acquired with an AxioCam MRm3 S/N 5762. Exposure time was 200 ms for both dyes and 20 ms for brightfield images.

2.2.3 Inhibition of LAP activity in C17-2 cells

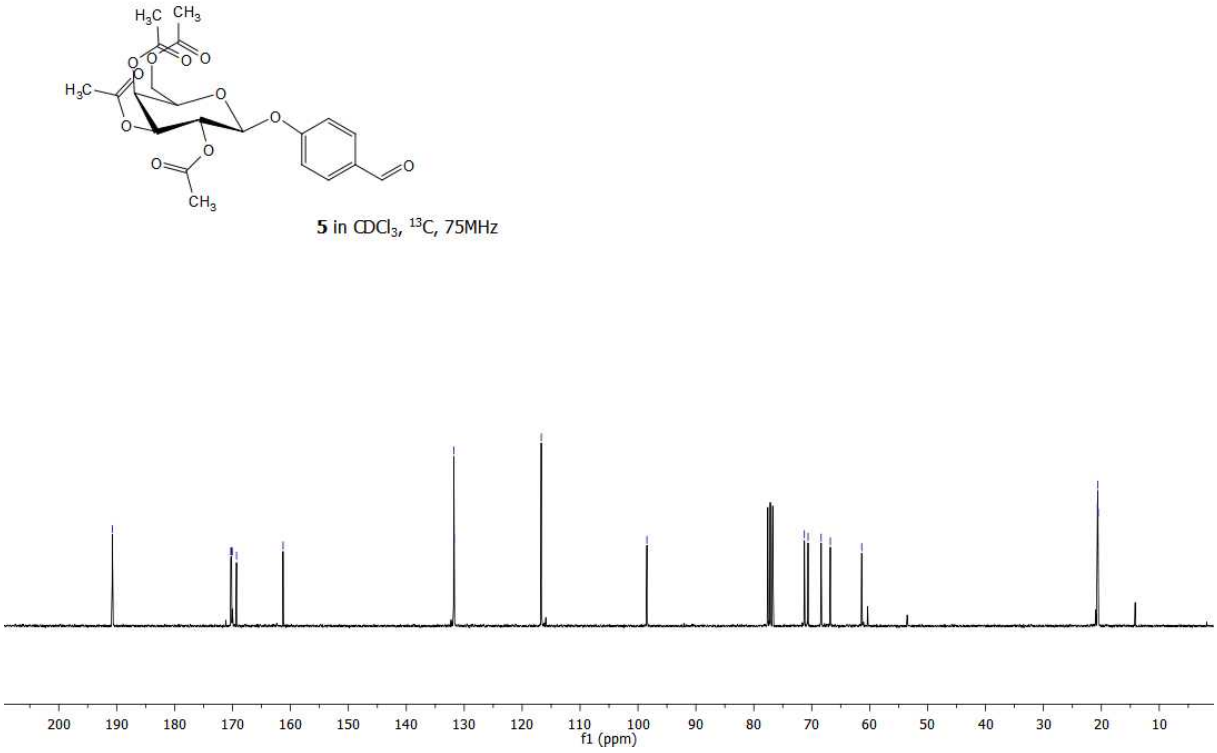
The same procedure was used as described in 2.2.2 but C17-2 cells were incubated with 5µM of L-Leucinethiol (oxidized dihydrochloride form, Sigma-Aldrich) during 1h before probe **1** (50µM) was added.

3 NMR spectra

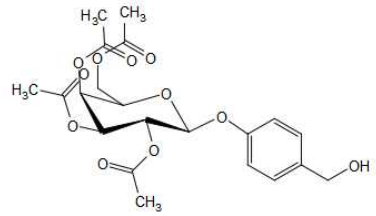
As a preamble, it should be noted that NMR spectra of compound **1** and **8** are very complex because of the presence of diastereoisomers (racemic aminomethylpiperidine spacer), conformers (piperidine chair) and rotamers (carbamate units). To confirm the structures, 2D experiments (COSY, HSQC, HMBC) were performed.



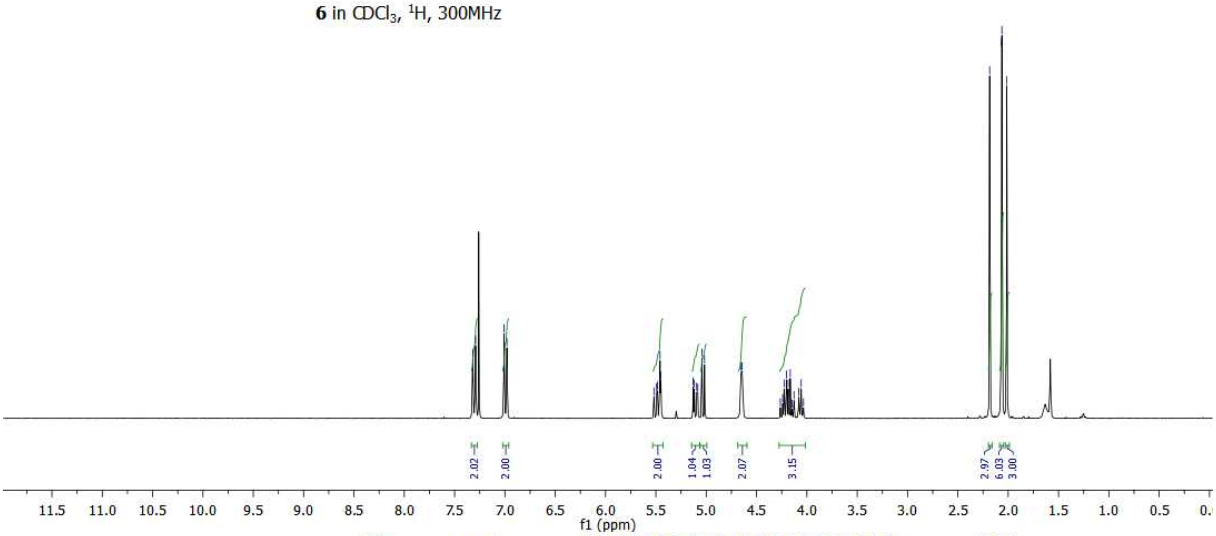
5 in $CDCl_3$, ^1H, 300MHz

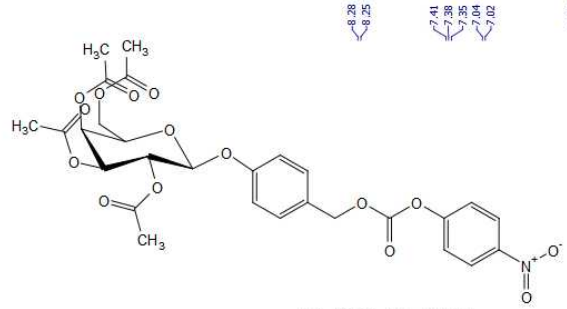


5 in $CDCl_3$, ^{13}C, 75MHz

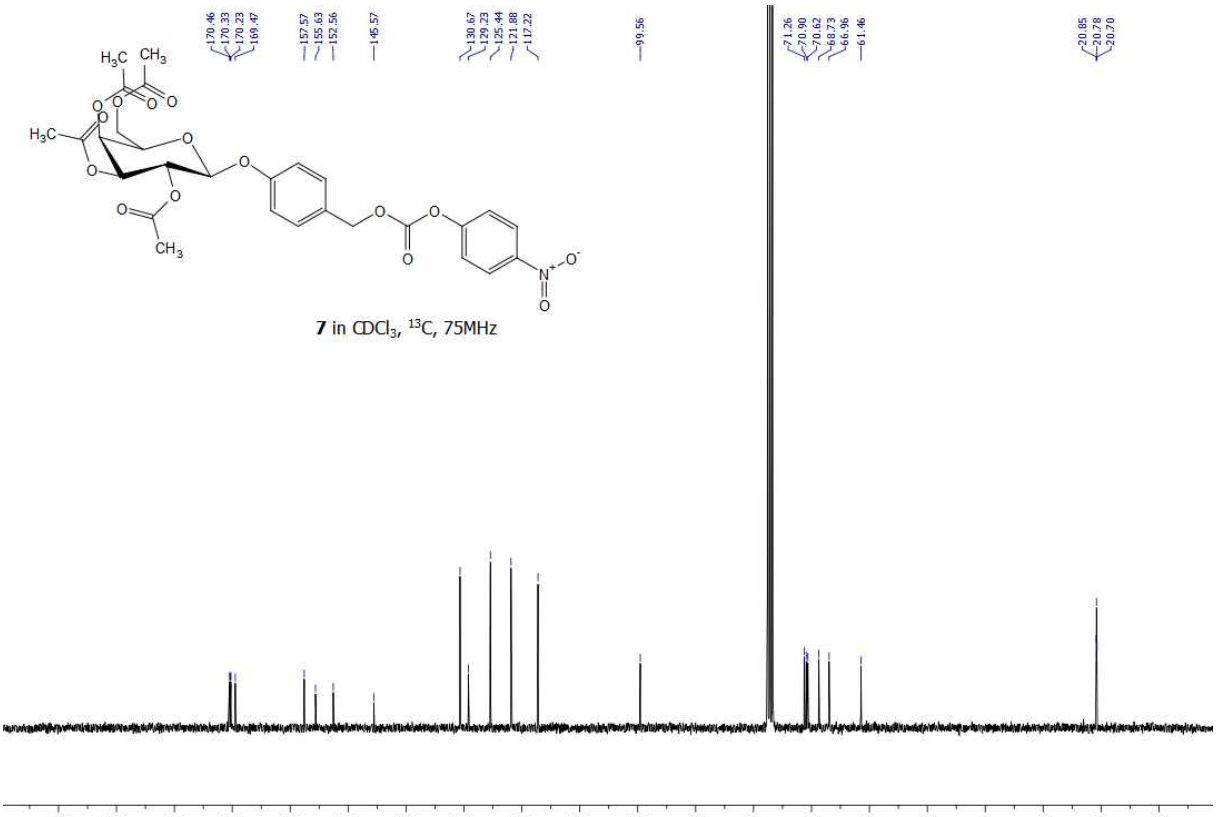


6 in $CDCl_3$, ^1H, 300MHz

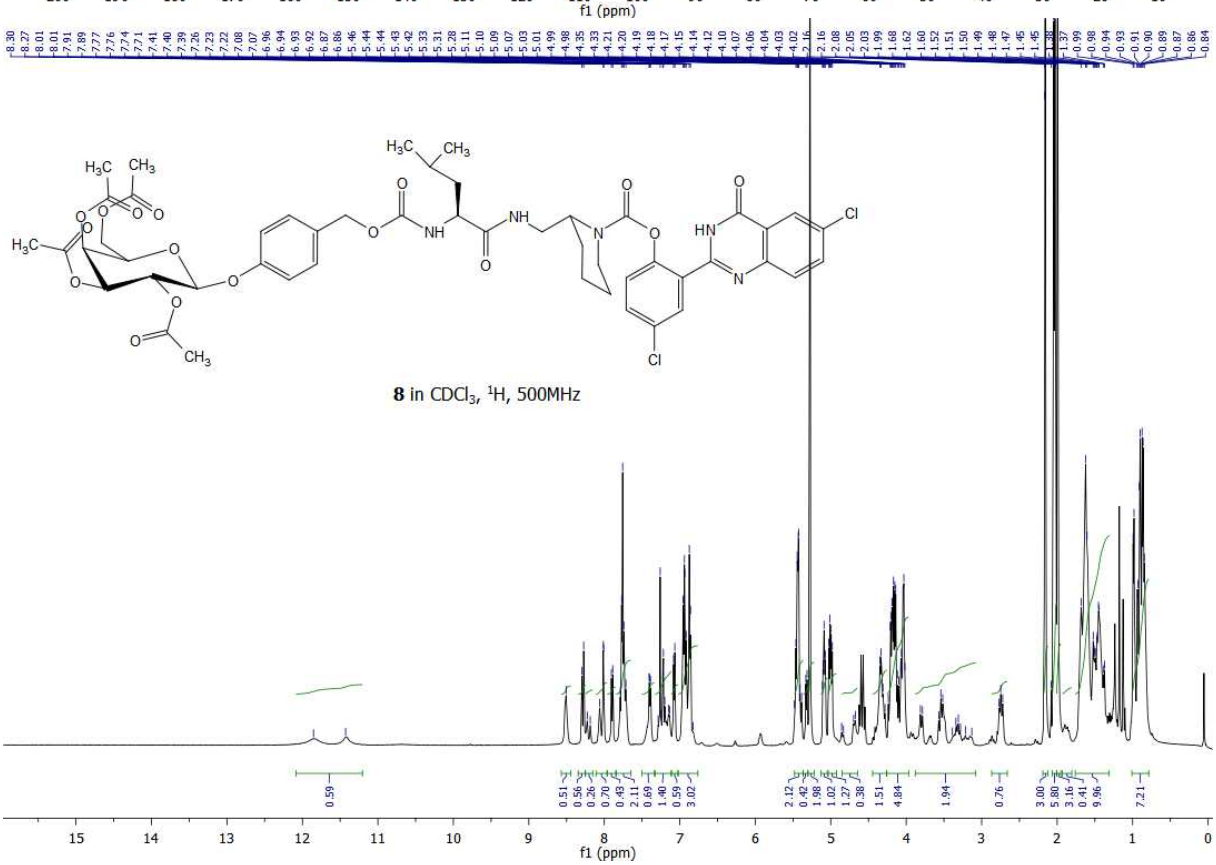




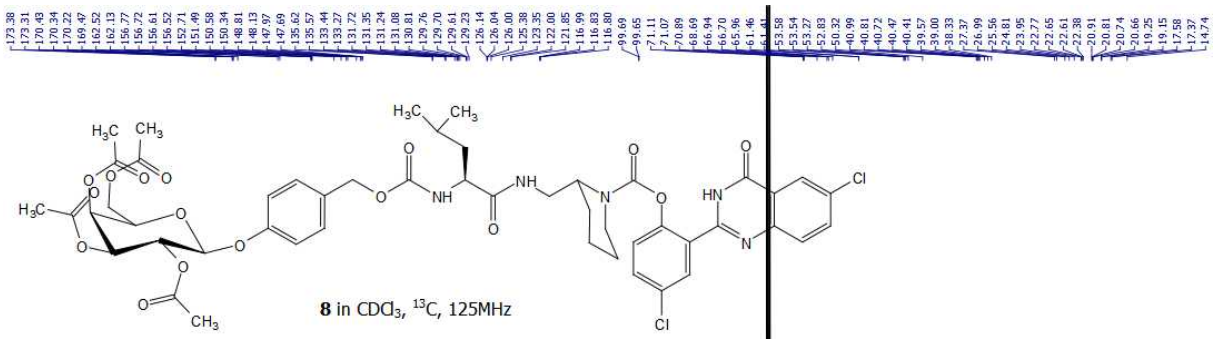
7 in $CDCl_3$, ^1H, 300MHz



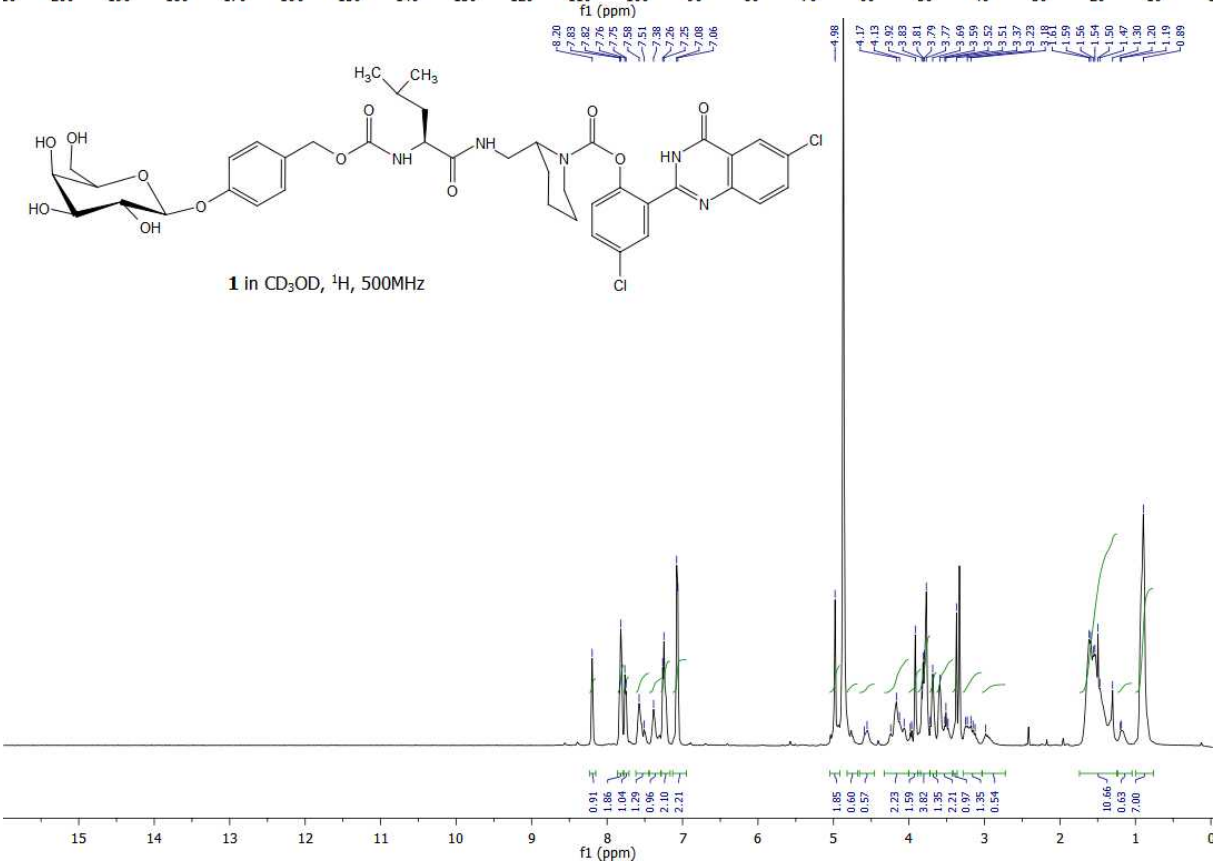
7 in $CDCl_3$, ^{13}C, 75MHz



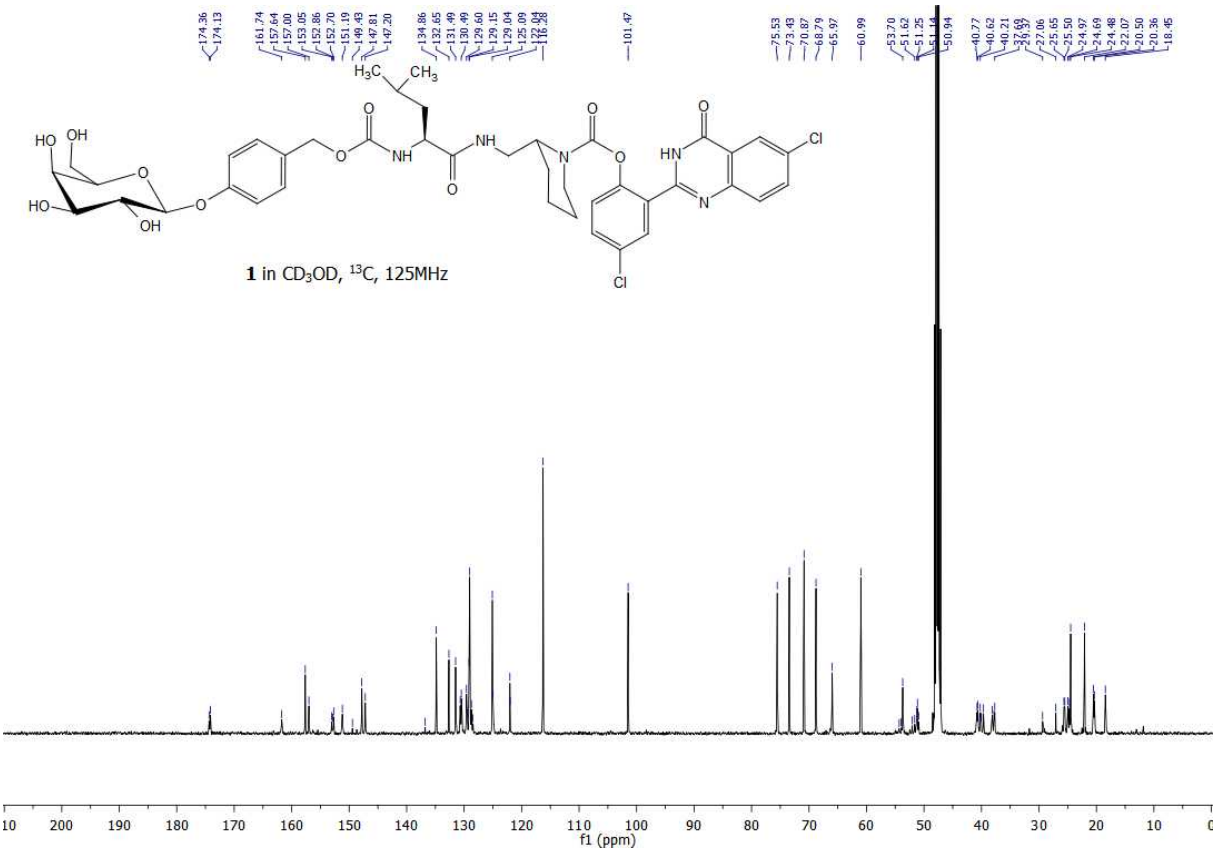
8 in $CDCl_3$, ^{1}H, 500MHz



8 in $CDCl_3$, ^{13}C, 125MHz



1 in CD_3OD, ^{1}H, 500MHz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f1 (ppm)

4 Reference

[1] T. Chauvin, P. Durand, M. Bernier, H. Meudal, B.-T. Doan, F. Noury, B. Badet, J.-C. Beloeil, E. Tóth, *Angew. Chem. Int. Ed. Engl.* **2008**, *47*, 4370–2.

[2] M. Prost, L. Canaple, J. Samarut, J. Hasserodt, *Chembiochem* **2014**, published online, doi: 10.1002/cbic.201402091.