Supplementary Information

PhenDV, a turn-off fluorescent quadruplex DNA probe for improving the sensitivity of drug screening assays

Claire Beauvineau,[a,b] Corinne Guetta,[a,b] Marie-Paule Teulade-Fichou[a,b] and Florence Mahuteau-Betzer* [a,b]

Table of Contents for Supplementary Information

1. Materials and reagents

Chemical reagents were purchased from Aldrich Sigma used without further purification.
Oligonucleotides purified by reversed-phase HPLC were purchased from Eurogentec. The dual fluorescently labeled oligonucleotides were purchased from Eurogentec. The donor fluorophore was 6-carboxyfluorescein (FAM) and the acceptor fluorophore was 6-carboxytetramethylrhodamine (TAMRA).

All chemicals were purchased from Sigma-Aldrich (USA) or Acros Organics (Belgium) and used as received. Preparative flash chromatographies were carried out with Merck silica gel (Si 60, 35–70 µm). NMR spectra were recorded on a Bruker Advance 300 spectrometer. Chemical shifts are reported in ppm downfield to TMS (δ = 0.00) and coupling constants are given in Hz. High-performance liquid chromatography was carried out on a Waters Alliance equipped with a photodiode array detector using an XterraMS column with a linear gradient of acetonitrile versus water containing 0.05% of TFA ranging from 2 to 30% over 5 min then to 100% over 9 min at a flow rate of 1 mL min^{-1}.
$PhenDC_3$, $PhenDC_6$, 360A, Pt-ttPy were synthesized as previously described.[1,2,3] Stock solutions of these ligands (2 mM in DMSO) were used for G4-FID assay, unless otherwise stated, and were stored at −20°C. Diethyl 4-pyridylmethylphosphonate, 1,10-phenanthroline-2,9-dicarbaldehyde, TO and cacodylic acid were purchased from Aldrich and used without further purification.
Stock solutions of TO and PhenDV (2 mM in DMSO) were used for fluorimetric titration, Job Plot, FRET melting assay and G4-FID assay. Fluorescent probe powders and solutions were stored and used, protected from light and used as aliquots to avoid freeze–thaw cycles. Fluorescence measurements (i.e., fluorimetric titrations, job plot and quantum yield) are performed on a

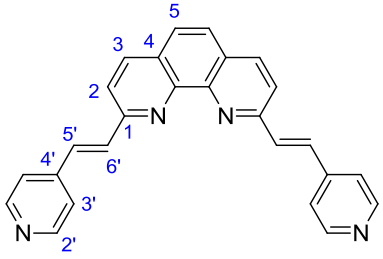
[1] Lemarteleur T, Gomez D, Paterski R, Mandine E, Mailliet P, Riou JF (2004) Stabilization of the c-myc gene promoter quadruplex by specific ligands' inhibitors of telomerase. Biochem Biophys Res Commun 323(3):802–808
[2] De Cian A, DeLemos E, Mergny J-L, Teulade-Fichou M-P, Monchaud D (2007) Highly efficient G-Quadruplex recognition by bisquinolinium compounds. J Am Chem Soc 129(7):1856–1857
[3] Bertrand H, Monchaud D, Cian AD, Guillot R, Mergny J-L, Teulade-Fichou M-P (2007) The importance of metal geometry in the recognition of G-quadruplex-DNA by metal-terpyridine complexes. Org Biomol Chem 5(16):2555–2559

Cary Eclipse Fluorescence spectrophotometer (Agilent Technologies). UV–Vis measurements were performed on a Cary Series UV-Vis spectrophotometer (Agilent Technologies). Fret melting measurements are performed on 7900HT Fast Real-Time PCR System (Applied Biosystems) with Microamp Fast optical 96-well reaction plate (Applied Biosystems). HT-G4-FID measurements were performed on a FLUOstar Omega microplate reader (BMG Labtech) with 96-well Non-Binding Surface black with clear bottom polystyrene microplates (Corning).

2. Synthesis

Synthesis of PhenDV

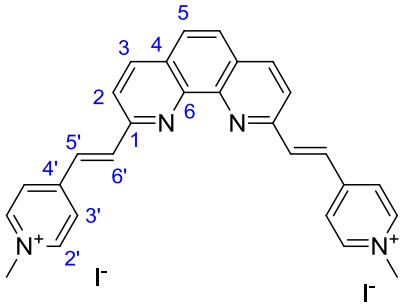
2,9-bis[(E)-2-(pyridin-4-yl)ethenyl]-1,10-phenanthroline 3



Diethyl 4-pyridylmethylphosphonate **1** (506 mg, 2.2 mmol) was dissolved in anhydrous THF (40 mL). NaH (95 mg, 2.4 mmol) was added at room temperature and the resulting solution was stirred for 15 min. 1,10-phenanthroline-2,9-dicarbaldehyde **2** (236 mg, 1 mmol) was added slowly to the mixture. The reaction was stirred protected from the light during 24h. After evaporation of the solvent, the crude product was purified by column chromatography on silica gel with dichloromethane/ethanol (100:0 -> 95:5, v/v) as eluent, giving **3** as a powder (71 mg, 18%).

CAS 1520076-96-3; **^{1}H NMR** (300 MHz, $CDCl_3$) δ 8.66 (4H, d, *J* = 6.0, 2'-H), 8.30 (2H, d, *J* = 8.5, 3-H), 7.98 (2H, d, *J* = 8.5, 2-H), 7.90 (2H, d, *J* = 16.5, 5'-H), 7.82 (2H, s, 5-H), 7.76 (2H, d, *J* = 16.5, 6'-H), 7.57 (4H, d, *J* = 6.0, 3'-H)

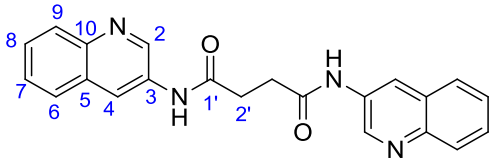
PhenDV



3 (71 mg, 0.183 mmol) was dissolved in a mixture CH_2Cl_2/MeOH (1:1, v/v) (5 mL). Iodomethane (0.5 mL, 8 mmol) was added. The reaction was heated at 35°C during 4h. Orange suspension was filtered and washed with Et_2O to give **PhenDV** as an orange solid (80 mg, 65%). **^{1}H NMR** (300 MHz, DMSO) : δ 8.99 (4H, d, J = 6.6, 2'-H), 8.66 (2H, d, J = 8.3, 3-H), 8.49 (4H,d, J = 6.6, 3'-H), 8.35 (2H, d, J = 16.2, 5'-H), 8.24 (2H, d, J = 16.2, 6'-H), 8.24 (2H, d, J = 8.3, 2-H), 8.09 (2H, s, 5-H), 4.34 ppm (6H, s, NMe) ; **^{13}C NMR** (75 MHz, DMSO) : δ 153.2 & 151.6 (C4' and C1), 145.5 (C6), 145.4 (C2'), 140.0 (C5'), 137.5 (C3), 129.0 (C4), 128.5 (C6'), 127.4 (C5), 124.5 (C3'), 123.4 (C2), 47.2 (NMe) ppm ; **HRMS (ESI-TOF)** : *m/z* calcd for $C_{28}H_{24}N_4I$ $[M]^+$: 543.1046; found 543.1039; **mp**>260°C

Synthesis of Bisquinolinium negative control $BisDC_3$

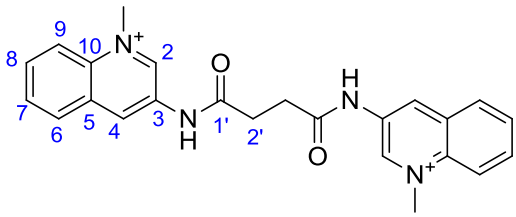
Compound 4



Succinyl chloride (400mg, 2.58mmol) in dry DCE (5mL) was added slowly over a period of 30 min to a stirred solution of 3-aminoquinoline (930mg, 2.5eq) containing TEA (0.90mL, 2.5eq) in dry DCE (20mL) under argon atmosphere at 20°C. The dark homogeneous reaction mixture was allowed to be stirred at r.t. under argon atmosphere overnight. The solvent was removed in vacuo at 45°C, then an aqueous solution of K_2CO_3 (10%w/v) was added to the crude material and the mixture was stirred for 2h. The resulting suspension was filtered and dried with Et_2O to yield **4** (145mg, 15%) as a colorless solid. Rf (DCM/EtOH 9:1) = 0.41; **^{1}H NMR** (300 MHz, DMSO-d6): δ= 10.56 (2H, s, NH), 8.92 (2H, d, J=2.0, 2-H), 8.71 (2H, d, J=2.0, 4-H), 7.94 (2H, d, J=8.0, 9-H), 7.89 (2H, d, J=7.5, 6-H), 7.62 (2H, t, J=8.0, 8-H), 7.54 (2H, t, J=7.5, 7-H), 2.82 (4H, s, 2'-H); **^{13}C NMR** (75 MHz, DMSO) : δ 171,3 (C1'),

144.4 (C10), 144.1 (C2), 133.0 (C3), 128.5 (C5), 127.9 (C7), 127.7 (C6 or C9), 127.6 (C6 or C9), 127.0 (C8), 121.6 (C4), 31.0 (C2') **LRMS (ESI-MS)** m/z = 371 $[M+H]^+$; **mp**>260°C

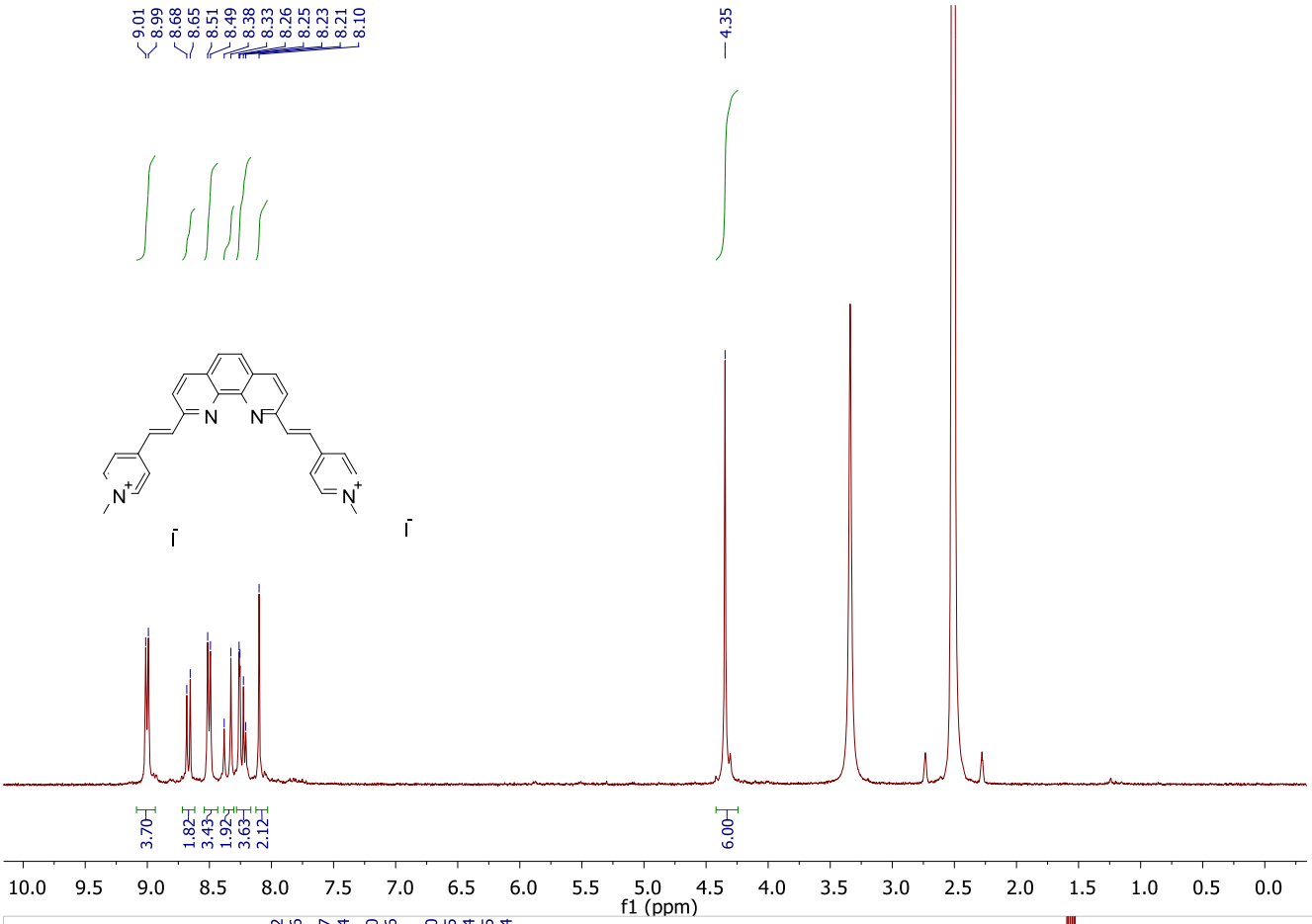
BisDC$_3$

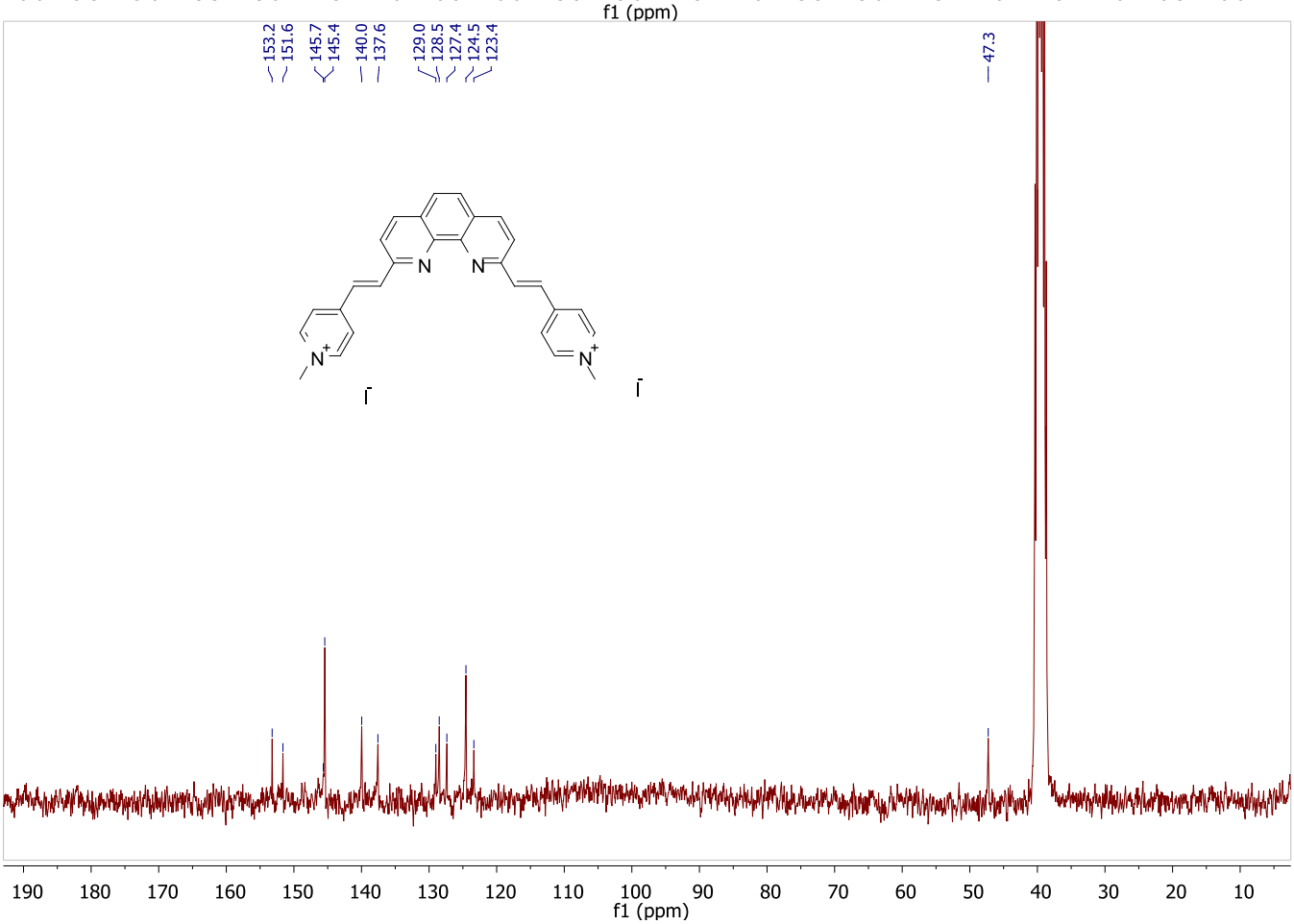


Methyl iodide (0.70mL, large excess) was added slowly to the solution of the Bis-quinolinium derivative (21mg, 0.057mmol) in dry DMF (0.70mL) at 40°C. The reaction mixture was kept stirring at 40°C overnight under argon atmosphere. The yellow suspension was filtered, washed with EtOH and dried with Et_2O to yield the expected compound **BisDC$_3$** (83mg, 78%) as a yellow solid (mixture of dimethylated and monomethylated compound 89/11).**^{1}H NMR** (300 MHz, DMSO-d6): δ= 11.23 (2H, s, NH), 9.63 (2H, bs, 2-H), 9.19 (2H, bs, 4-H), 8.45 (4H, m, 6-H and 9-H), 8.15 (2H, m, 8-H), 7.99 (2H, m, 7-H), 4.66 (6H, s, NMe), 2.92 (4H, s, 2'-H); **^{13}C NMR** (75 MHz, DMSO) : δ 171,6 (C1'), 143.4 (C2), 135.2 (C3), 133.5 (C10), 133.2 (C8), 132.2 (C4), 130.1 (C5), 129.7 (C7), 129.3 (C6), 119.0 (C9), 46.2 (NMe), 30.5 (C2'); **LRMS (ESI-MS)**: m/z =200 $[M-2I]^+$; **HRMS (ESI)**: *m/z* calcd for $C_{24}H_{24}IN_4O_2$ $[M-I]^+$: 527.0944; found 527.0946; **mp**>260°C

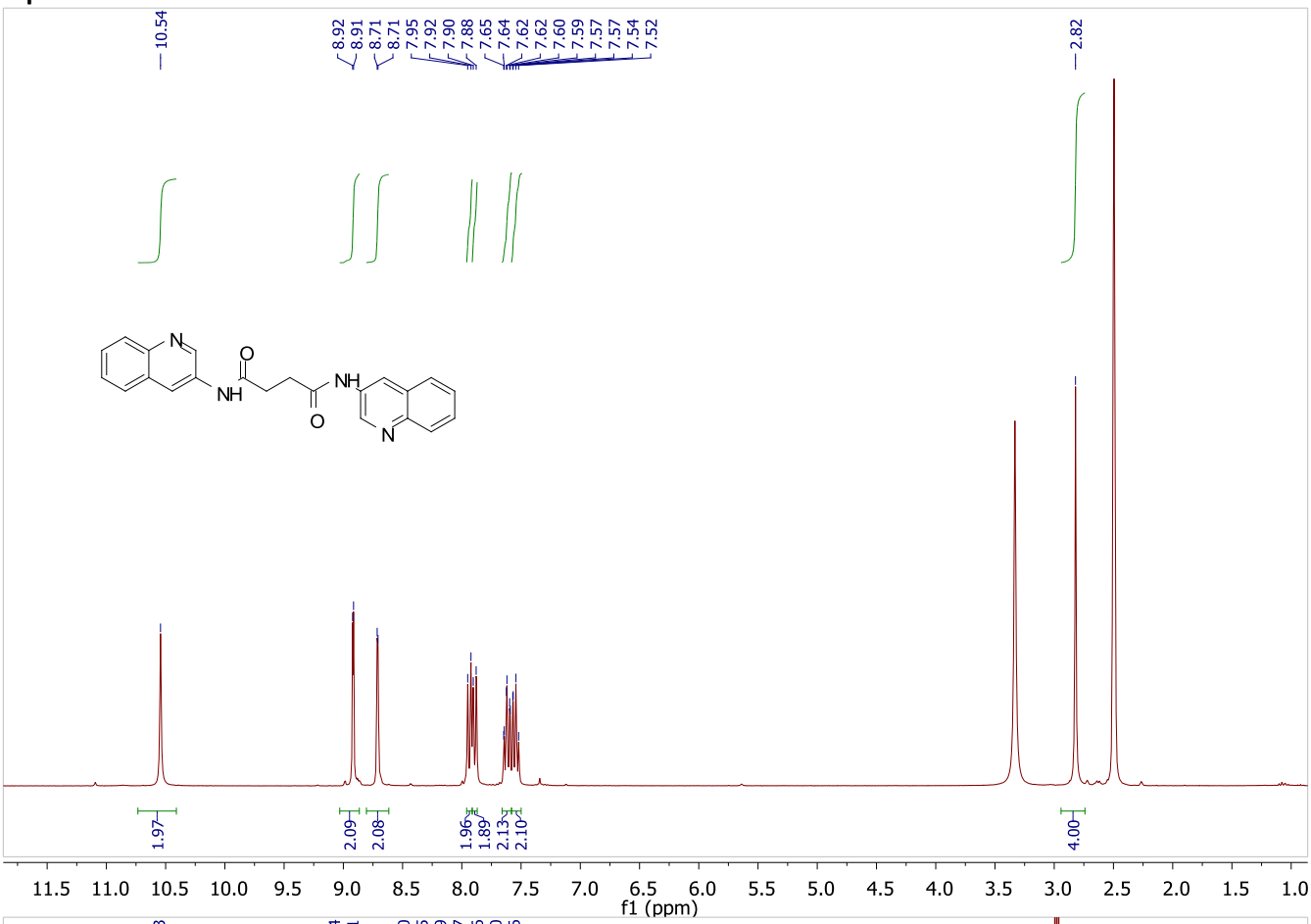
3. NMR spectra and LC-MS chromatograms

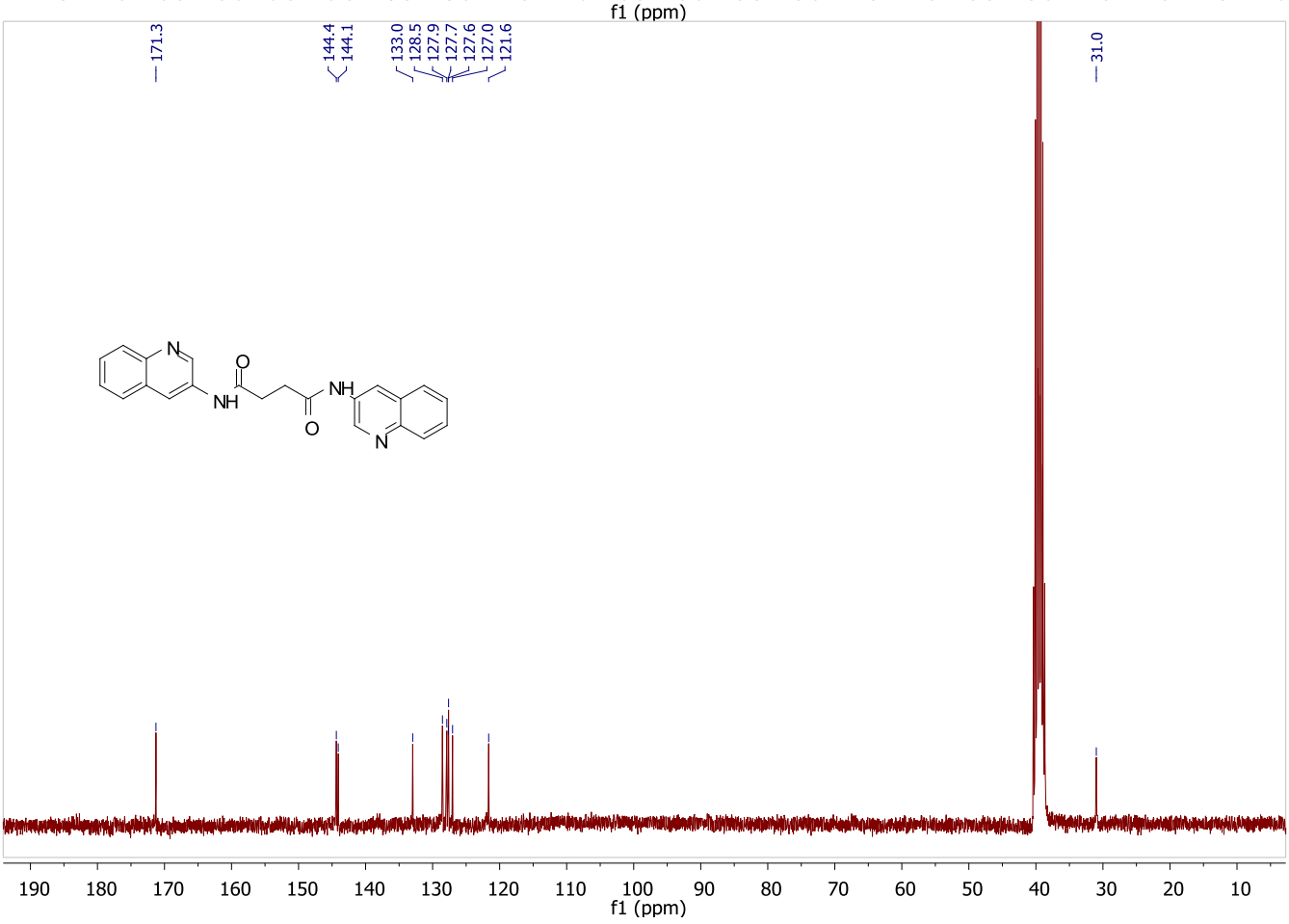
PhenDV

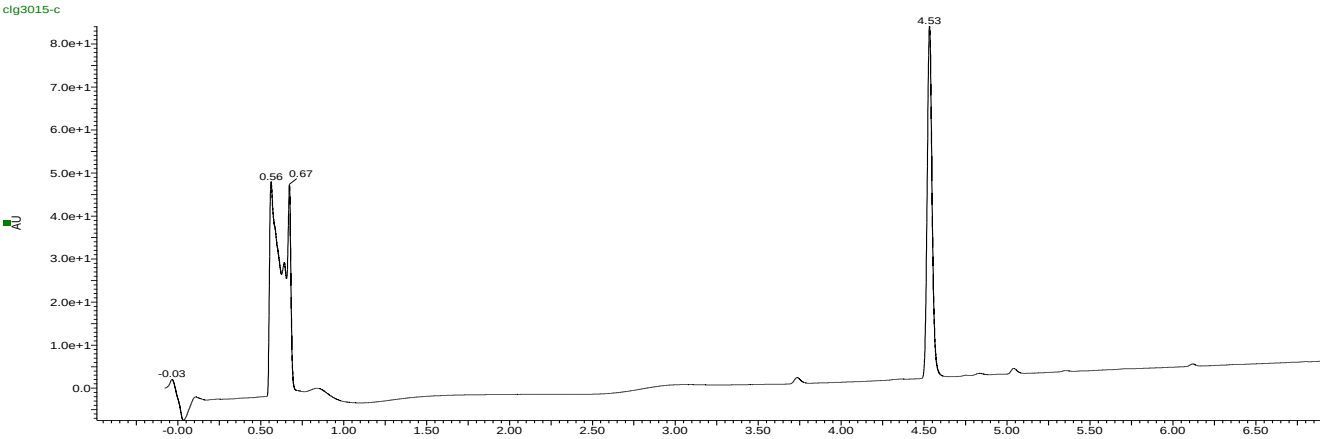


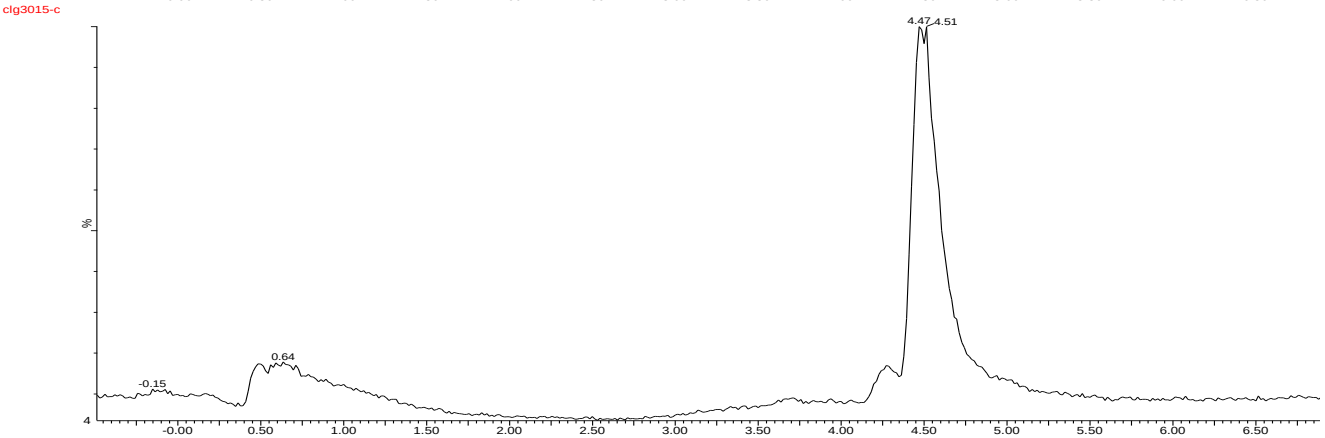


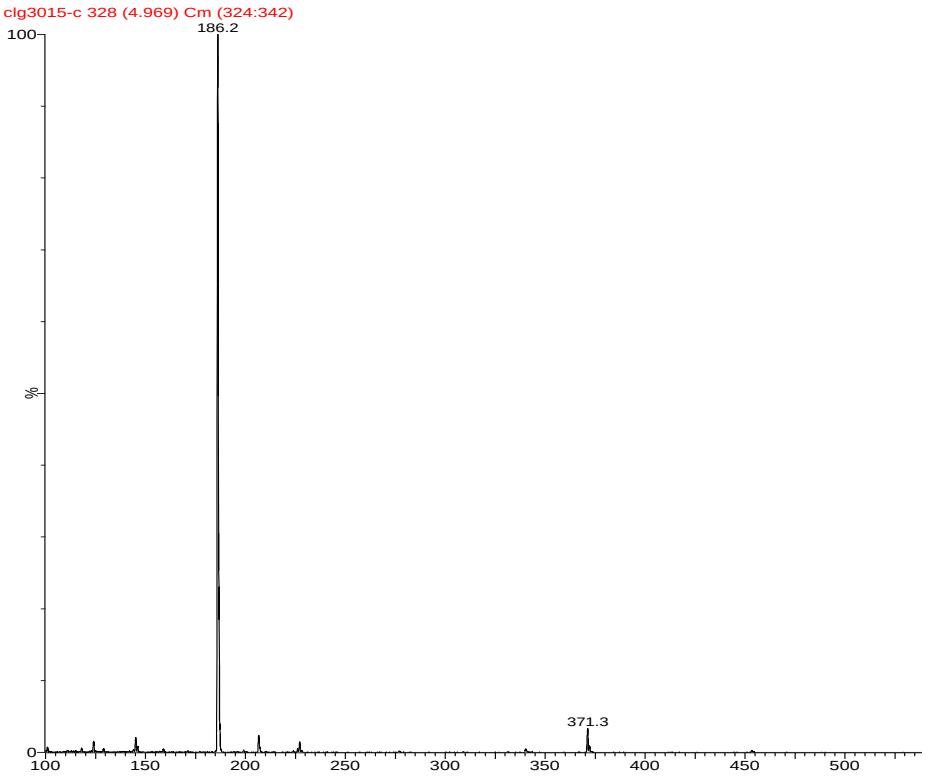
Compound 4


10.54
8.92
8.91
8.71
8.71
7.95
7.92
7.90
7.88
7.65
7.64
7.62
7.62
7.60
7.59
7.57
7.57
7.54
7.52
2.82
1.97
2.09
2.08
1.96
1.89
2.13
2.10
4.00
f1 (ppm)

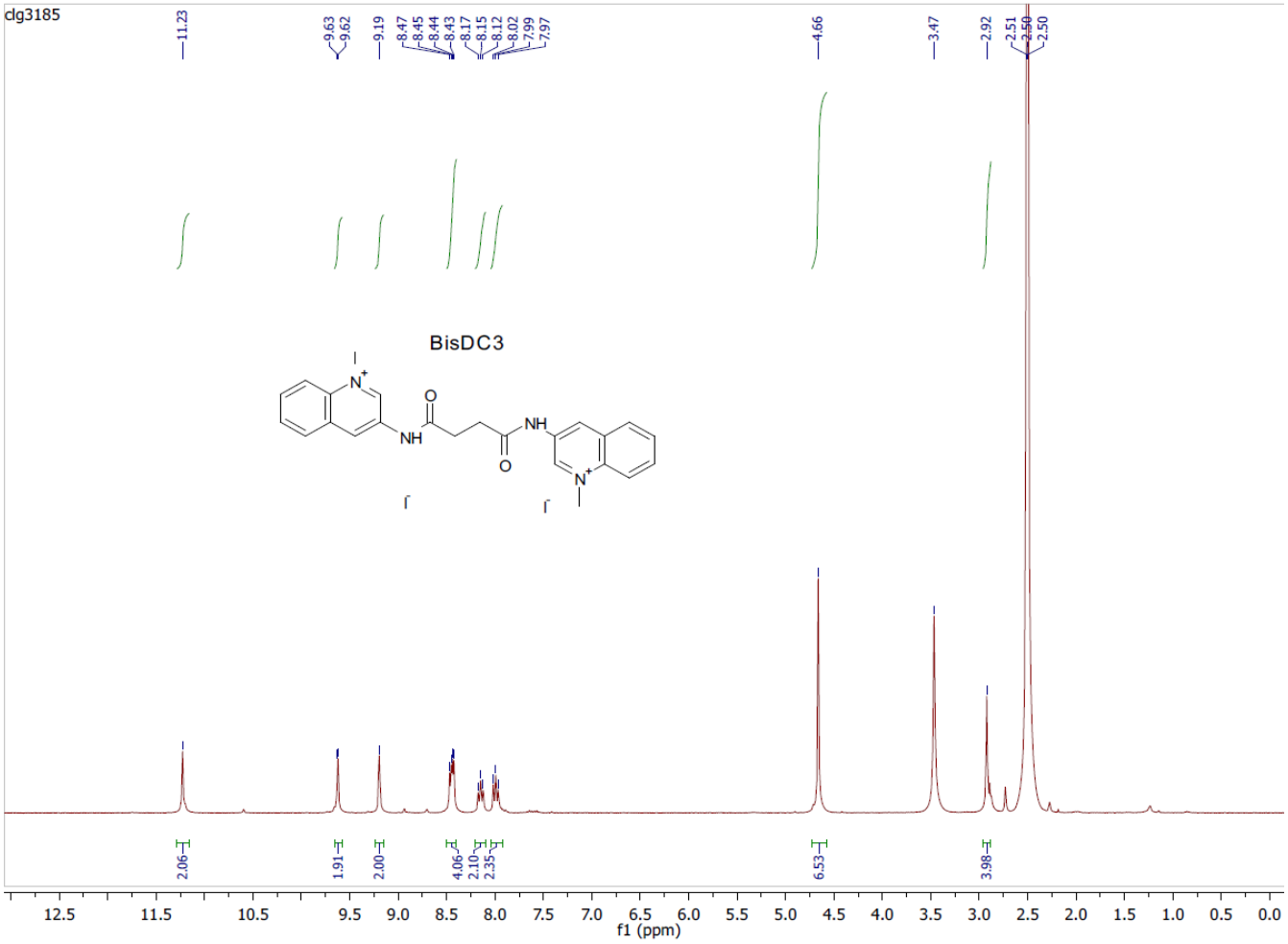

171.3
144.4
144.1
133.0
128.5
127.9
127.7
127.6
127.0
121.6
31.0
f1 (ppm)

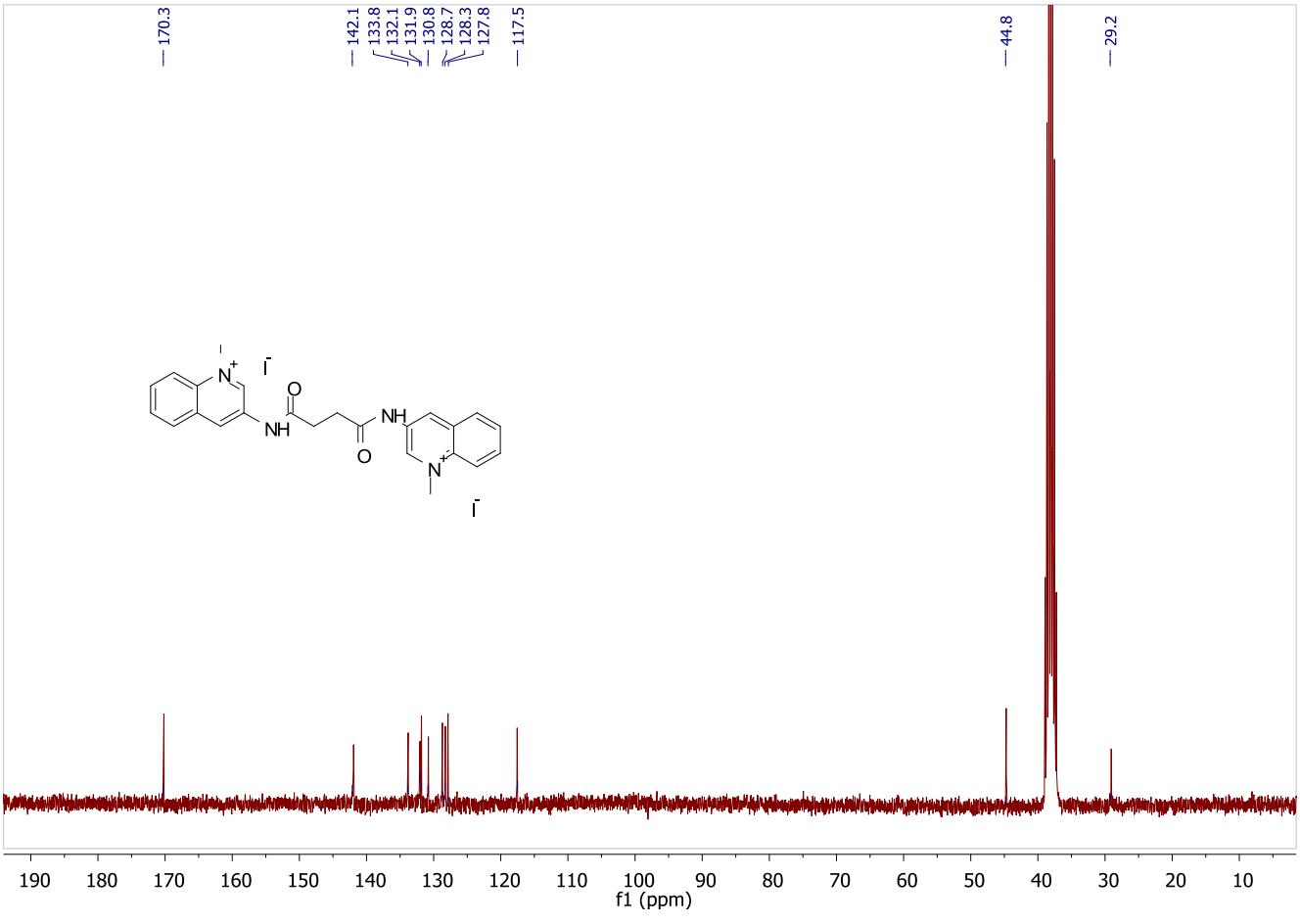

clg3015-c
AU
8.0e+1
7.0e+1
6.0e+1
5.0e+1
4.0e+1
3.0e+1
2.0e+1
1.0e+1
0.0
-0.03
0.56
0.67
4.53
-0.00
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50
5.00
5.50
6.00
6.50

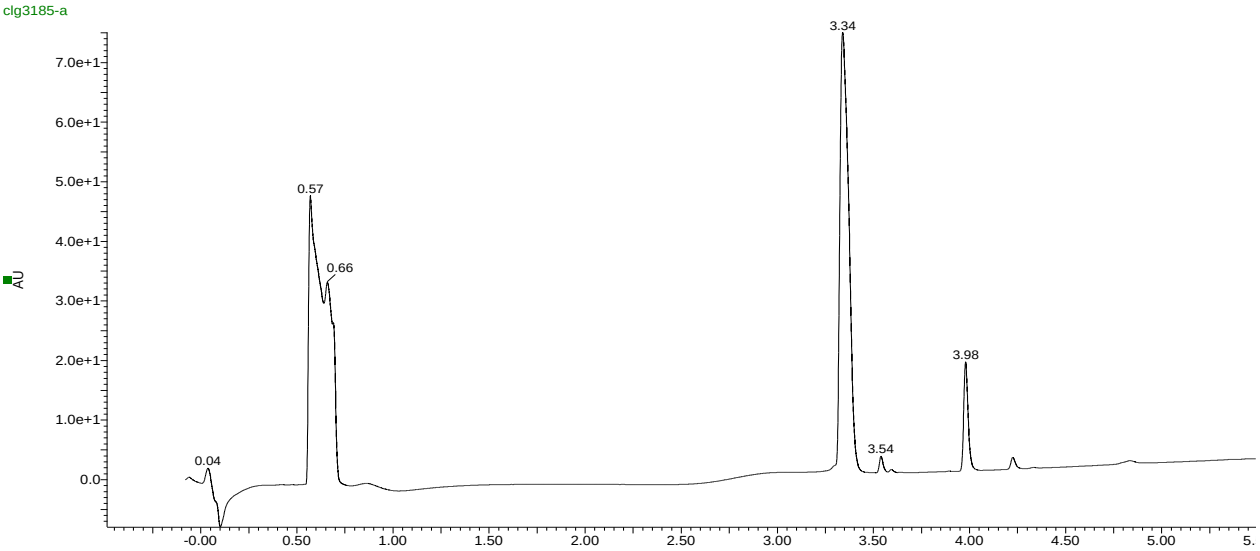

clg3015-c
%
4.47
4.51
0.64
-0.15
4
-0.00
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50
5.00
5.50
6.00
6.50

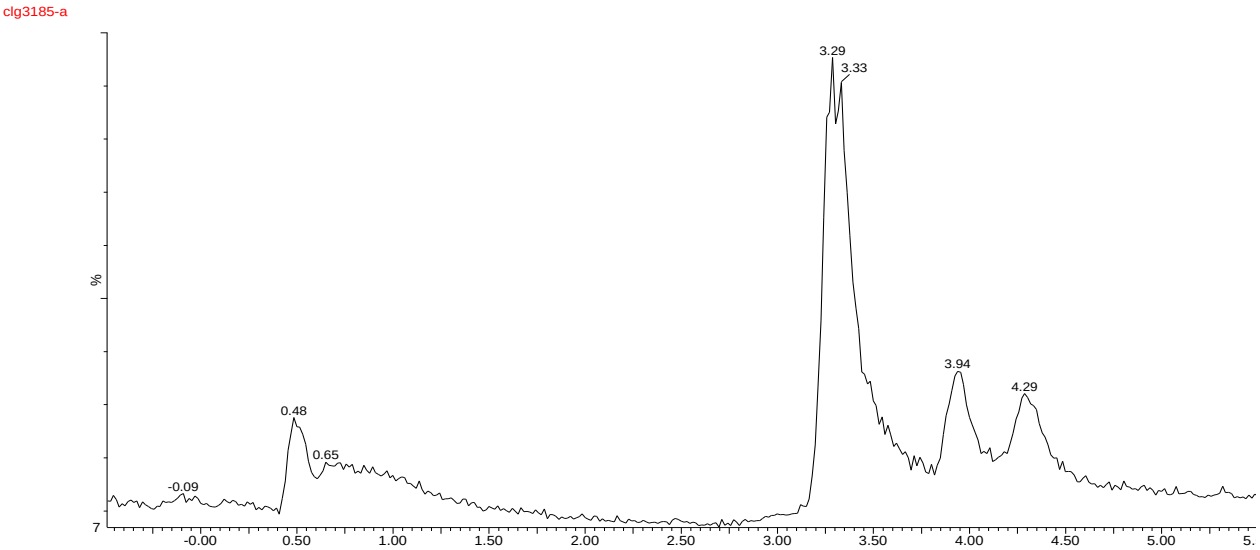

clg3015-c 328 (4.969) Cm (324:342)
100
%
0
186.2
371.3
100
150
200
250
300
350
400
450
500

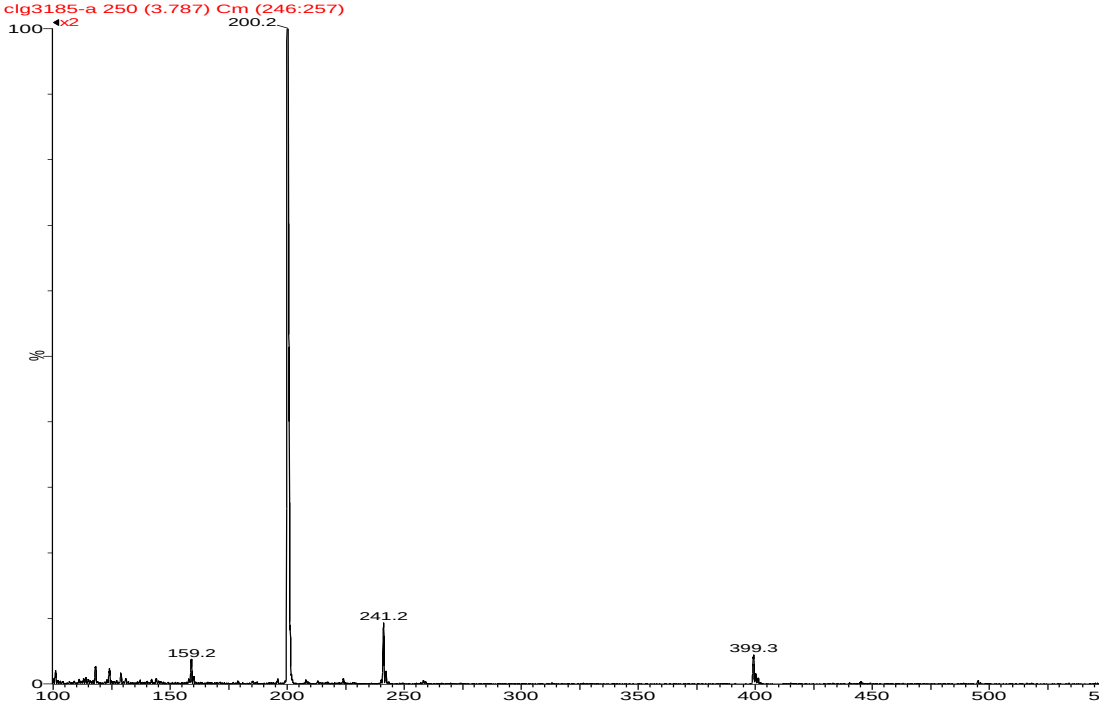
BisDC3


dg3185
11.23
9.63
9.62
9.19
8.47
8.45
8.44
8.43
8.17
8.15
8.12
8.02
7.99
7.97
4.66
3.47
2.92
2.51
2.50
2.50
BisDC3
2.06
1.91
2.00
4.06
2.10
2.35
6.53
3.98
f1 (ppm)


170.3
142.1
133.8
132.1
131.9
130.8
128.7
128.3
127.8
117.5
44.8
29.2
f1 (ppm)


clg3185-a
AU
7.0e+1
6.0e+1
5.0e+1
4.0e+1
3.0e+1
2.0e+1
1.0e+1
0.0
0.04
0.57
0.66
3.34
3.54
3.98
-0.00
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50
5.00


clg3185-a
%
7
-0.09
0.48
0.65
3.29
3.33
3.94
4.29
-0.00
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50
5.00


clg3185-a 250 (3.787) Cm (246:257)
100
x2
%
0
200.2
241.2
159.2
399.3
100
150
200
250
300
350
400
450
500

4. Spectroscopic Measurements

Preparation of oligonucleotides

For fluorometric titrations, oligonucleotides were dissolved in K^+100 buffer (10 mM lithium cacodylate buffer pH 7.3, 100 mM KCl). For G4-FID assay, oligonucleotides were dissolved in K^+100* buffer (10 mM lithium cacodylate buffer pH 7.3, 100 mM KCl, 1% DMSO). Oligonucleotide concentrations were determined on the basis of their absorbances at 260 nm.

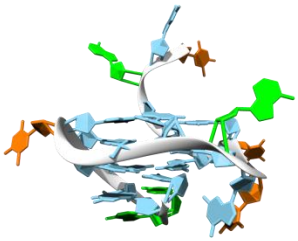
For FRET melting assay, oligonucleotides were dissolved in K^+1 buffer (10 mM lithium cacodylate buffer pH 7.3, 1 mM KCl, 99 mM LiCl) except for F-21-T. F-21-T was dissolved in K^+10 buffer (10 mM lithium cacodylate buffer pH 7.3, 10 mM KCl, 90 mM LiCl).

Prior to use, all oligonucleotides were pre-treated by heating at 95°C for 5 min, then rapidly cooled to 4 °C to favor the intramolecular folding by kinetic trapping. Duplex-DNA ds26 was prepared by heating the self complementary strand at 90°C for 5 min in K^+100 buffer followed by a slow cooling over 6 h.

Oligonucleotides sequences

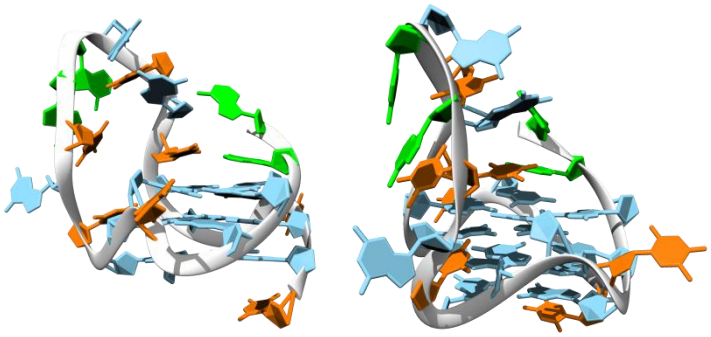
- 22AG : 5'-A**GGG**TTA**GGG**TTA**GGG**TTA**GGG**-3'

- PU24T : 5'-TGAGGGTGGTGAGGGTGGGGAAGG-3'



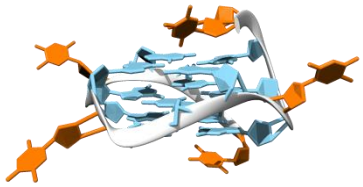
W. J. Chung, B. Heddi, F. Hamon, M. P. Teulade-Fichou, A. T. Phan, Angewandte Chemie, 2014, 999

- c-kit1 : 5'-**GGG**A**GGG**CGCT**GGG**AGGA**GGG**-3'
- CEB25-WT : 5'- AAGGGTGGGTGTAAGTGTGGGTGGGT-3'



S. Amrane, M. Adrian, B. Heddi, A. Serero, A. Nicolas, J.-L. Mergny, A. T. Phan J. Am. Chem. Soc., **2012**, 5807

- CEB25-L111(T) : 5'-AAGGGTGGGTGGGTGGGT-3'



N. Q. Do, A. T. Phan, *Chemistry a European Journal* 2012, 14752

- ds26 : 5'-CAATCGGATCGAATTCGATCCGATTG-3'

F-21-T : 5'-FAM-GGGTTAGGGTTAGGGTTAGGG-TAMRA-3'
F-PU24T-T : 5'-FAM -TGAGGGTGGTGAGGGTGGGGAAGG-TAMRA-3'
F-CEB25-WT-T : 5'-FAM-AAGGGTGGGTGTAAGTGTGGGTGGGT-TAMRA-3'
F-CEB25-L111(T)-T : 5'-FAM-AAGGGTGGGTGGGTGGGT-TAMRA-3'

Measurement of fluorescence quantum yield (Φ_F)

Fluorescence quantum yields (Φ_F) of free PhenDV sample was calculated using quinine sulfate (in 0.5 M H_2SO_4 solution) as a standard (Φ=0.54). Emission spectra of PhenDV solutions were recorded from 380 to 700 nm with excitation at 370 nm. Absorbance (optical density, OD) of all the samples was recorded at 370 nm, and quantum yields were calculated according to Equation (1), in which Φ_{ref} is the quantum yield of the reference, A_{sample} and A_{ref} are the areas under the emission spectra of the sample and the reference, respectively, and OD_{ref} and ODs_{ample} are the absorbances of the reference and the sample, respectively, measured at the excitation wavelength; n_{sample} and n_{ref} are the refractive indices of the reference and the sample, respectively, in solution.

$$\varphi_{sample} = \varphi_{ref}\left(\frac{A_{sample}}{A_{ref}}\right) \times \left(\frac{OD_{ref}}{OD_{sample}}\right) \times \left(\frac{n_{ref}}{n_{sample}}\right)^2 \quad (1)$$

Fluorescence quantum yields (Φ_F) of TO in the presence of different DNA samples were calculated using rhodamine (EtOH solution) as a standard (Φ=0.92). Emission spectra of 1 µM TO and quinine solutions were recorded from 511 to 700 nm with excitation at 501 nm. Oligonucleotides were added to the TO solution to give a final concentration of 6 µM. Absorbance (optical density, OD) of all the samples was recorded at 511 nm, and quantum yields were calculated according to Equation (1).

Fluorimetric titrations

A temperature of 20°C was kept constant with a thermostated cell holder. Each titration was performed in a 1-ml quartz cell, in K^+100-buffer in a total volume of 1 ml. Titrations were performed with a solution of the fluorescent probe (TO; 0.5 µM or PhenDV; 0.5 or 1 µM) in the corresponding buffer in which gradual addition of oligonucleotides was carried out (up to 10 molar equivalents). After each addition, a fluorescence emission spectrum was recorded at 501, or 387 nm excitation wavelength, respectively. The fluorescence emission area was measured between 510–750, or 397–700 nm, respectively, with 1.0 nm increment, 0.1 s integration time, 3/3 nm (excitation/emission) slits. The titration curves were obtained by plotting the fluorescence emission area enhancement against the oligonucleotide concentration. Fluorimetric titrations were performed according to published procedures[4] and the binding constants were determined by fitting of the experimental data to the theoretical model:

$$\frac{I}{I_0} = 1 + \frac{Q-1}{2}\left(A + xn + 1 - \sqrt{(A + xn + 1)^2 - 4xn}\right) \quad (2)$$

where $Q = I_\infty/I_0$ is the minimal fluorescence intensity in the presence of excess ligand;
n is the number of independent binding sites per quadruplex;
$A = 1/(K_b \times c_L)$;
$x = c_{G4}/c_L$ is the titration variable.

FRET-Melting experiments

Stabilization of compounds with quadruplex-structure was monitored via FRET-melting assay performed in 96-well plates on real time PCR apparatus 7900HT Fast Real-Time PCR System as follow: 5 min at 25°C, then increase of 0.5°C every minute until 95°C. Each experimental condition was tested in duplicated in a volume of 25 µL for each sample. FRET-melting assay was performed with five dual fluorescently labeled DNA oligonucleotide sequences oligonucleotides. The donor fluorophore was 6-carboxyfluorescein, FAM, and the acceptor fluorophore was 6-carboxytetramethylrhodamine, TAMRA. The 96-well plates (Applied Biosystems) were prepared by aliquoting the annealed DNA (0.2 µM in potassium cacodylate buffer) into each well, followed by 1 µL of the ligand (100 µM (5 eq) or 20 µM (1 eq) in DMSO). For competition experiments, duplex competitors were added to 200 nM quadruplex sequences at final concentrations of 3.0 µM (15 eq) and 10.0 µM (50 eq), with a total reaction volume of 25 µL, with the labeled oligonucleotide (0.2 µM) and the ligand (1.0 µM). Measurements were made with excitation at 492 nm and detection at 516 nm. The change in the melting temperature at 1.0 µm compound concentration—ΔTm (1.0 µM)—was calculated from at least two experiments by subtraction of the blank from the averaged melting temperature of each compound (1.0 µM). Final analysis of the data was carried out using Origin Pro 8.6 data analysis.

HT-G4-FID assay

Each G4-FID assay is performed in a 96-well Non-Binding Surface black with clear bottom polystyrene microplates (Corning). Every ligand is tested on a line of the microplate, in duplicate (that is four ligands tested by microplate). The microplate is filled with (a) K^+100* solution (qs for 200 µL) (b) 10 µL of a solution of pre-folded oligonucleotides (5 µM) and fluorescent probe (TO (10 µM – 2 eq) or PhenDV (7.5 µM – 1.5 eq)) and (c) an extemporaneously prepared 5 µM ligand solution in K^+100* buffer (0 to 100 µL along the line of the microplate, i.e., from column A to column H: 0, 0.125, 0.25, 0.375, 0.5, 0.625, 0.75, 1.0, 1.25, 1.5, 2.0 and 2.5 µM; see Electronic Supplementary Material for more details). After 5 min of orbital shaking at 500 rpm, fluorescence is measured using the following experimental parameters; positioning delay: 0.5 s, 20 flashes per well, emission/excitation filters

[4] K. Jäger, J. W. Bats, H. Ihmels, A. Granzhan, S. Uebach and B. O. Patrick, *Chem. Eur. J.*, **2012**, *18*, 10903-10951

for TO: 485/520, PhenDV : 355/520, gain adjusted at 80% of the fluorescence from the most fluorescent well (i.e., a well from column A for TO and a well from column H).

The percentage of TO displacement is calculated from the fluorescence intensity (F), using:

$$\%\ TO\ displacement = 1 - \frac{F}{F_0}$$

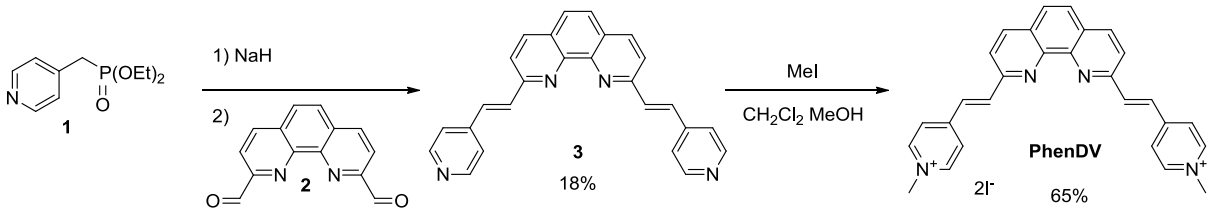
F_0 being the fluorescence from the fluorescent probe bound to DNA without added ligand.

In the case of PhenDV, the fluorescence of the unbound probe is not negligible. The percentage of displacement becomes:

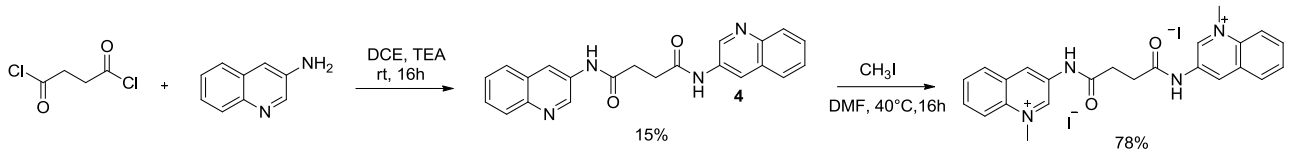
$$\%\ PhenDV\ Displacement = \frac{F - F_0}{F_{ligand+probe} - F_0}$$

The PhenDV displacement is calculated from the fluorescence intensity F, $F_{(ligand+probe)}$ which refers to the fluorescence of the probe in presence of the ligand (without G4) and F_0 being the fluorescence without added ligand. The term $F_{(ligand+probe)}$ is necessary as the ligand can quench the fluorescence of the probe. The percentage of displacement is then plotted as a function of the concentration of added ligand. The DNA affinity was evaluated by the concentration of ligand required to decrease the fluorescence of the probe by 50%, noted DC50, and determined after non-linear fitting of the displacement curve.

5. Figure S1: Synthesis of PhenDV



6. Figure S2: Synthesis of BisDC$_3$



7. Figure S3: Fluorimetric titrations

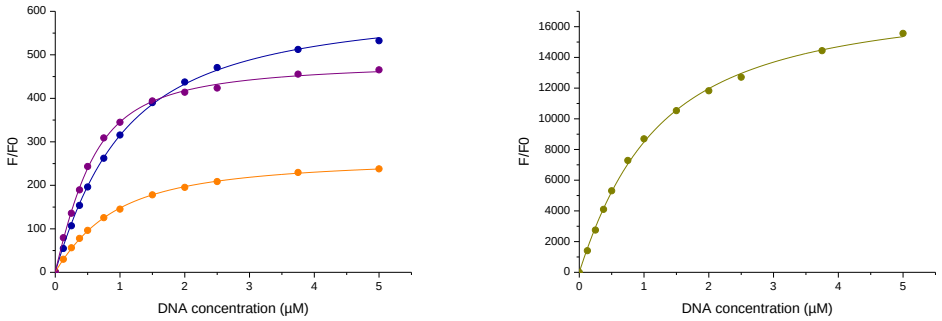


Figure S3a : Fluorimetric titrations of TO with 22AG (blue royal), PU24T (orange), CEB25-WT (green), CEB25-L111(T) (purple), fluorimetric titration with ckit1 was already described[5]

[5] E. Largy, F. Hamon and M. P. Teulade-Fichou, *Analytical and bioanalytical chemistry*, 2011, **400**, 3419-3427.

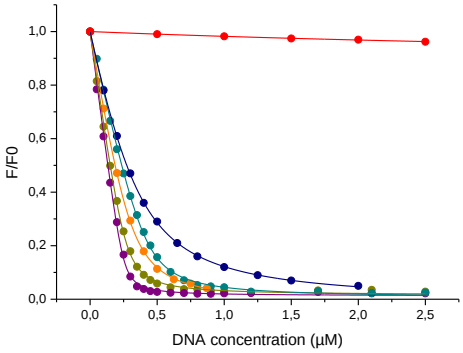
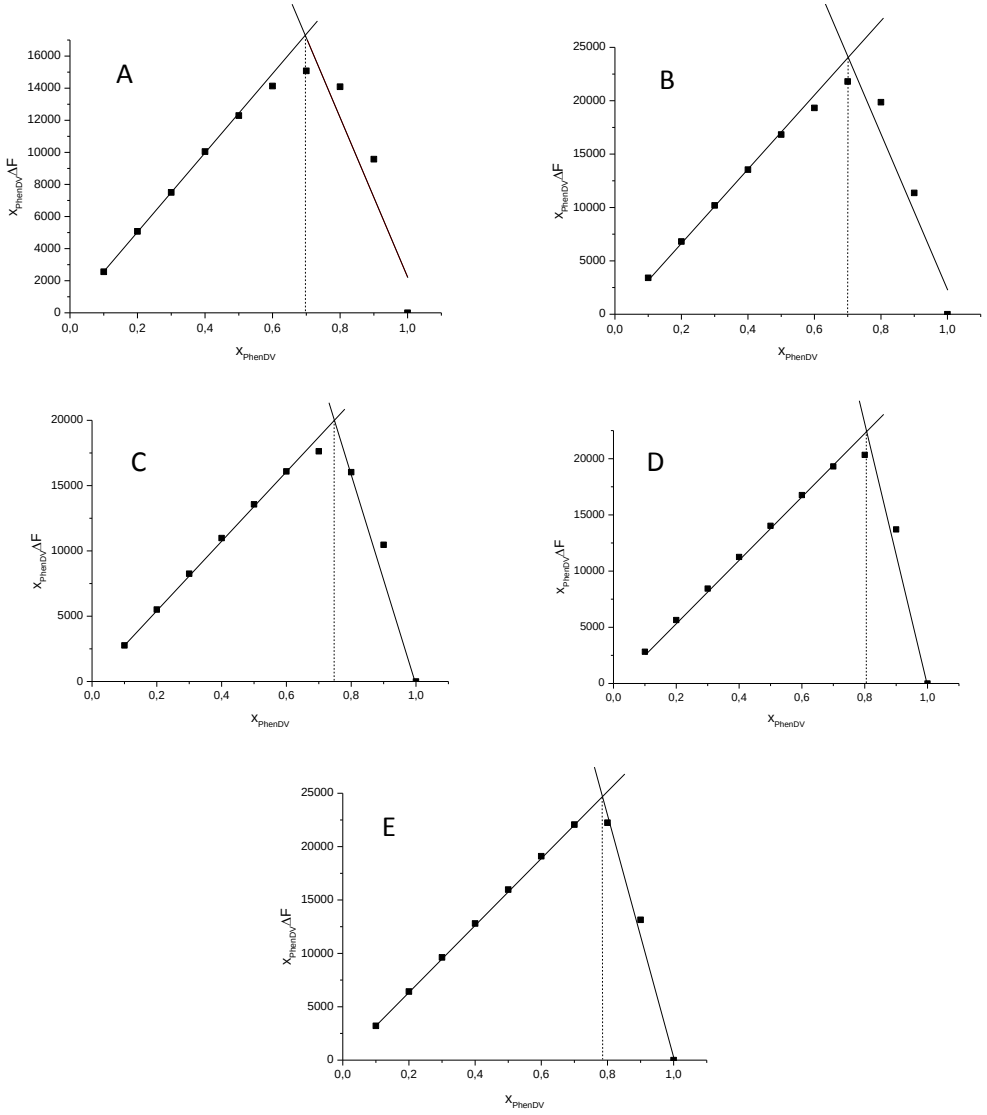


Figure S3b : Fluorimetric titrations of PhenDV (0.5 µM) with 22AG (blue royal), PU24T (orange) and ds26 (red) & Fluorimetric titrations of PhenDV (1µM), CEB25-WT (green), CEB25-L111(T) (purple), ckit1 (dark cyan)

8. Figure S4: Job Plot



Jobs' plot obtained from fluorimetric analysis of mixtures of PhenDV with 22AG (A), PU24T (B), Ckit1 (C), CEB25-WT (D) and CEB25-L111(T) (E) in K^+100 buffer ([PhenDV] + [DNA] = 2 µM). $\Delta F = F_0 - F_{exp,}$ where F_0 is the maximal fluorescence intensity in the presence of an excess of PhenDV. X_{PhenDV}= molar fraction of PhenDV. λ_{ex}=387 nm/λ_{em}=397-700 nm

9. Figure S5: FRET Melting

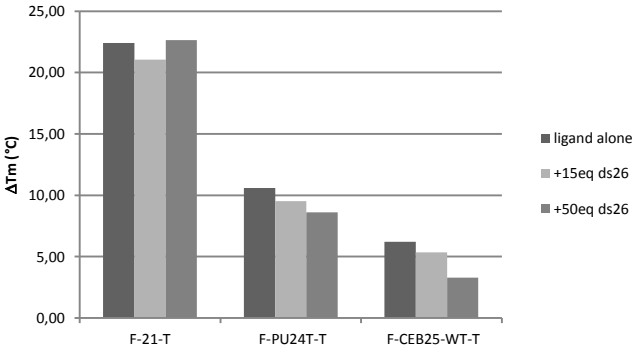


Figure S5a: FRET-melting stabilization by PhenDV (5 eq) (ΔTm in °C) of the labeled F-21-T sequence (0.2 µM) in K^+10 buffer, the labeled F-PU24T-T, F-CEB25-WT-T sequence (0.2 µM) in K^+1 buffer, in the presence of increasing concentrations of competitor ds26 (0, 15 and 50 equivalents)

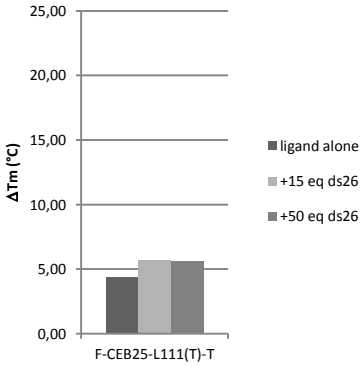


Figure S5b: FRET-melting stabilization by PhenDV (1 eq) (ΔTm in °C) of the labeled F-CEB25-L111(T)-T sequence (0.2 µM) in K^+10 buffer, in the presence of increasing concentrations of competitor ds26 (0, 15 and 50 equivalents)

10. Figure S6: G4-FID Plots

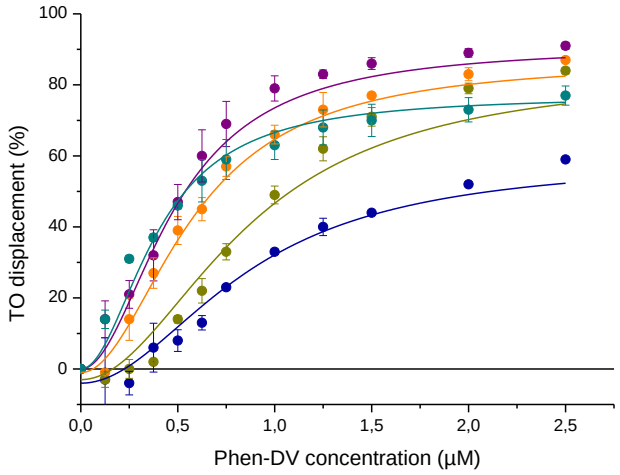


Figure S6a: TO displacement by PhenDV with 22AG (blue royal), PU24T (orange), CEB25-WT (green), CEB25-L111(T) (purple) and ckit1 (dark cyan) 0,25 µM in buffer K^+100*

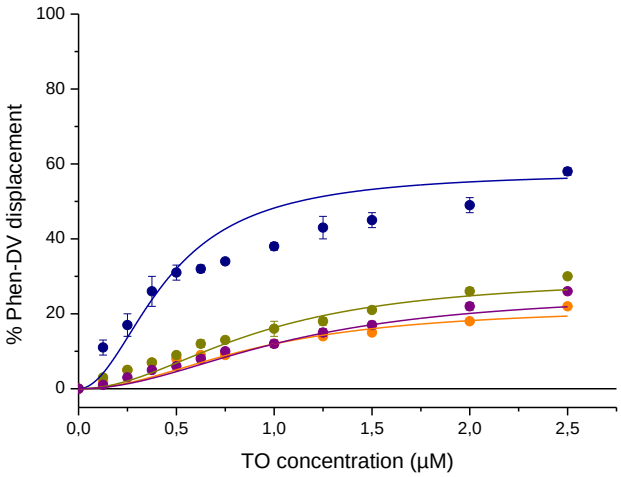


Figure S6b: PhenDV displacement by TO with 22AG (blue royal), PU24T (orange), CEB25-WT (green), CEB25-L111(T) (purple) and ckit1 (dark cyan) 0,25 µM in buffer K^+100*

11. Figure S7: HT-G4-FID Plots

PhenDC$_3$ (red), 360A (blue), Pt-ttPy (magenta), BisDC$_3$ (green)