Supplementary Material (ESI) for Organic & Biomolecular Chemistry

Supplementary Information for

A hydrophilic three side-chained triazatruxene as a new strong and selective G-quadruplex ligand

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Organic & Biomolecular Chemistry

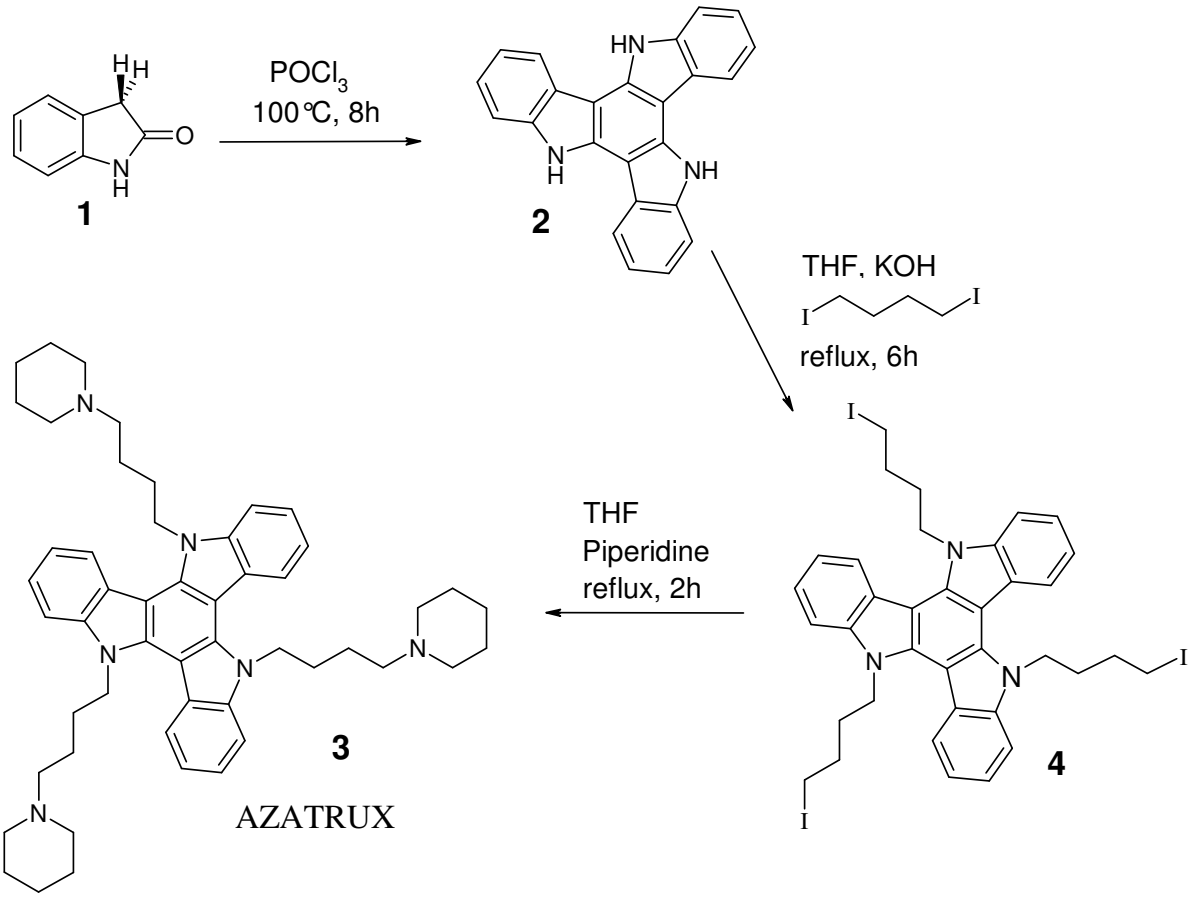
I. Materials and instrumentation:

2-indolinone and 1,4-diiodobutane were purchased from Fluka; the other commercial reagents and solvents were purchased from Aldrich. TLC glass plates (silica gel 60 F_{254}) and silica gel 60 (0.040-0.063 mm) were purchased from Merck. ^{1}H and ^{13}C-NMR spectra were performed with Varian Gemini 200 and Varian Mercury 300 instruments. Elemental analyses (C, H, N) were carried out on EA1110 CHNS-O (CE instruments). UV/Vis absorbition spectra were performed on a JASCO V-530 spectrophotometer. MES-KCl buffer is composed by 10 mM MES at pH 6.5 and 50 mM KCl.
Single-stranded oligonucleotides were purchased from Eurofins MWG Operon (Ebersberg, Germany). Their sequences are as follows: TG_4T (5'-TGGGGT-3'); F21T (5'-GGGTTAGGGTTAGGGTTAGGG-3'); DK66 (5'-CGCGAATTCGCG-3'). Calf thymus DNA, used in competition experiments, was purchased from Sigma-Aldrich and subjected to sonication (Sonyprep 150 sonicator) for 8 minutes to obtain an average length of 500bp (according to gel electrophoresis analysis with Mass Ruler DNA ladder mix-low range).

II. Molecular modelling simulations:

Molecular modelling simulations were performed using the InsightII package and the X-ray structure of the 22-mer human telomeric DNA sequence AGGG(TTAGGG)3 (PDB code 1KF1).[25] The binding site was defined as the external 3' G-quartet plane.[8] Initially 200 ligand orientations were randomly centred on the G-quadruplex structure, charges were not considered and the complexes were minimized for 500 steps. The 75 lowest energy structures were then subjected to simulated annealing, then 500 steps of minimization was followed by molecular dynamics over a period of 10 ps. The resulting structures were then minimized.

III. Detailed scheme for the preparation of AZATRUX:



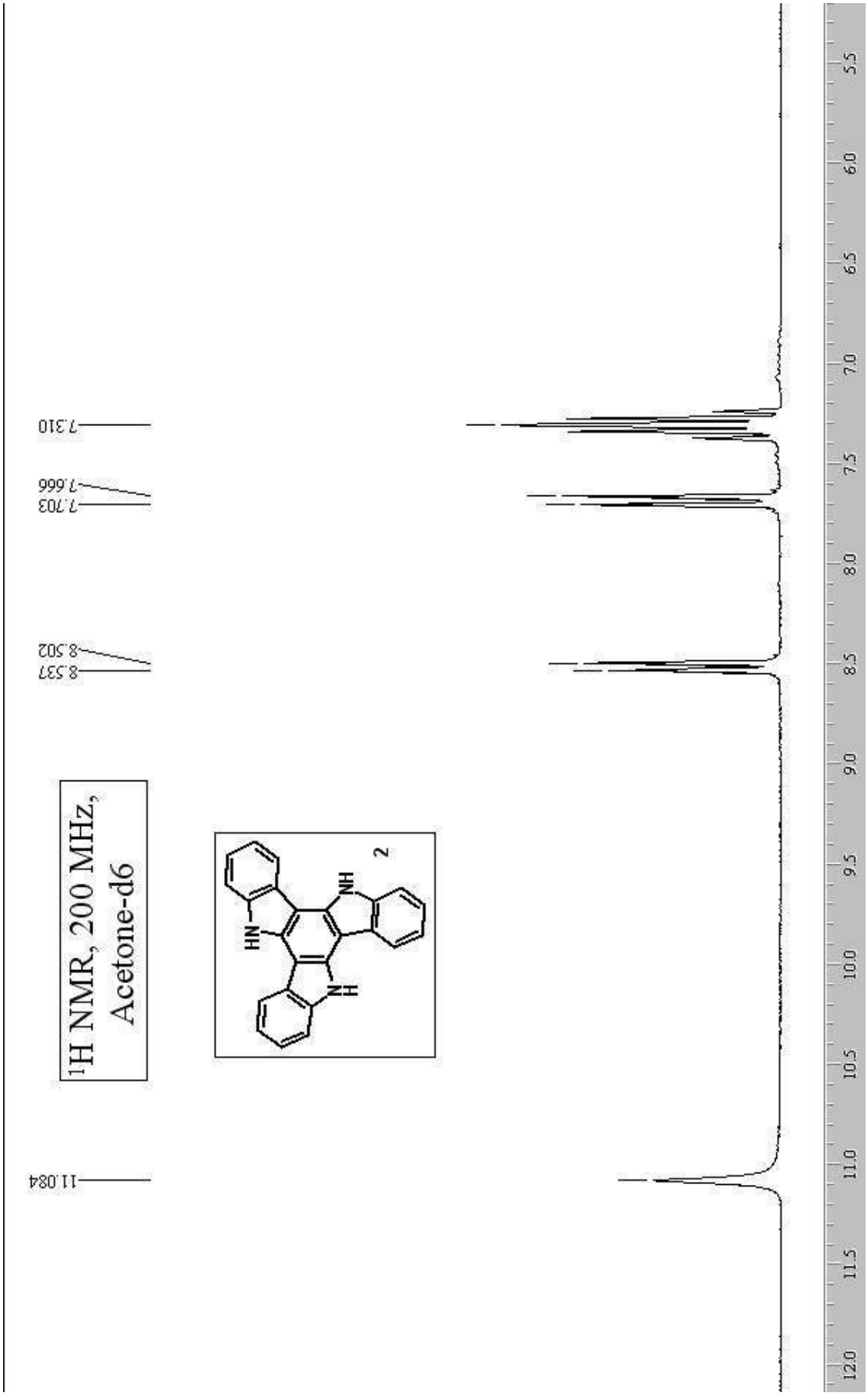
10,15-dihydro-5H-diindolo[3,2-a:3',2'-c]carbazole (2): A mixture of 2-indolinone **1** (2.0 g, 15 mmol) and 10 ml of $POCl_3$ was heated at 100°C for 8 h. Then, the reaction mixture was poured into ice and neutralized carefully with KOH until pH 7-8. After neutralization, the precipitate was filtered to give the crude product as a brown solid. The latter was dissolved in methanol, adsorbed on silica gel and purified by flash chromatography (ethyl acetate/n-hexane = 15:85) to give the pure compound of formula **2** as a pale yellow solid (830 mg, yield 48%).

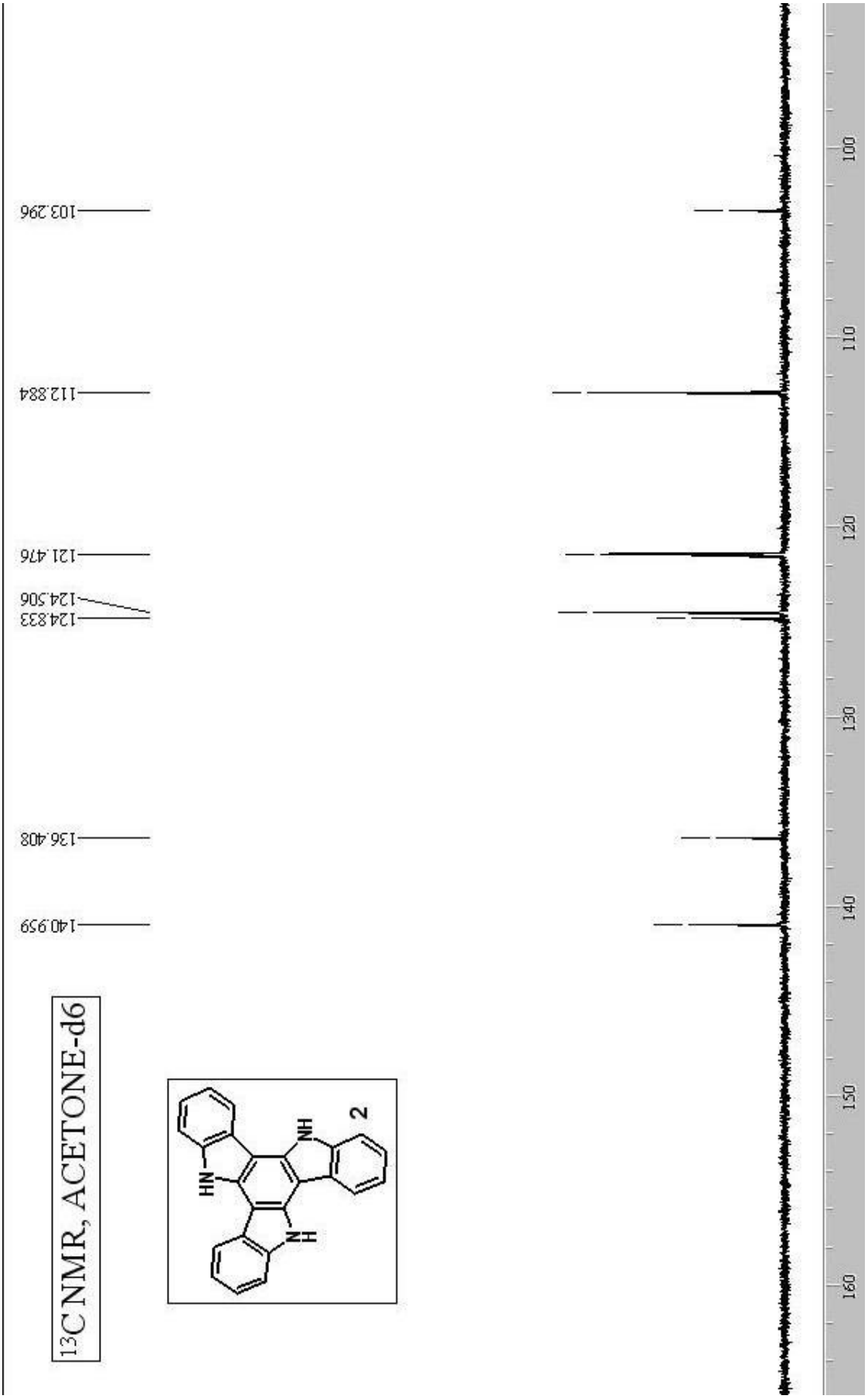
^{1}H NMR (200 MHz, acetone-d6) δ 11.08 (1s, 3H, N_{arom}-H), 8.52 (1d, 3H, J=7, aromatic H), 7.68 (1d, 3H, J= 7.4, aromatic H), 7.31 (1m, 6H, aromatic H) ppm; ^{13}C NMR (acetone-d6) δ: 141.0, 136.4, 124.8, 124.5, 121.5, 121.4, 112.9, 103.3 (8 aromatic C) ppm; UV (DMSO) λ max, nm (ε x10^{-4}, $M^{-1}cm^{-1}$) : 274 (3.3), 309 (6.8), 325 (3.6) , 342 (1.5); MS (ESI) m/z 344.01 [$(M-H)^-$] (Calcd for $C_{24}H_{14}N_3$: 344.40).

5,10,15-tris[4(1-piperidino)butyl]diindolo[3,2-a:3',2'-c]carbazole (3, AZATRUX): A mixture of **2** (355 mg, 1.03 mmol) and KOH (576 mg, 10.3 mmol) in THF (20 ml) was heated under reflux for 10 min. 1,4-Diiodobutane (2.0 ml, 15 mmol) was then added and the mixture was heated under reflux for 6h. The mixture was diluted with AcOEt, washed with 10% aqueous HCl and then with brine solution. The organic layer was dried with Na_2SO_4 and evaporated under vacuum. The crude product was dissolved in chloroform, adsorbed on silica gel and purified by flash chromatography (ethyl acetate/n-hexane = 5:95) to give 5,10,15-tris(4-iodobutyl)diindolo[3,2-a:3',2'-c]carbazole (**4**, 373 mg, yield 40%) as a dark yellow viscous oil. A mixture of this intermediate compound (209 mg, 0.23 mmol) and piperidine (0.69 ml, 7.0 mmol) was heated in THF (5ml) under reflux for 2h. The mixture was evaporated under reduced pressure and purified by flash chromatography (ethyl acetate saturated with 30% aqueous ammonia solution) to give the compound of formula **3** (161 mg, yield 91%) as a dark yellow viscous oil. The compound **3** (110 mg) was precipitated in the form of its hydrochloride salt by dissolving it in a mixture of methanol/HCl (methanol/aqueous HCl 37%=95:5) and adding diethyl ether: 81 mg of a white solid was obtained with a yield of 58%. ^{1}H NMR (300 MHz, D_2O) δ 7.61 (d, 3H, J=7.8, aromatic H), 7.40 (m, 6H, aromatic H), 7.25 (m, 3H, aromatic H), 3.98 (m, 6H, N_{arom}-CH_2), 2.89 (br, 6H, $N_{piperidine}$-CH_2), 2.53 (m, 6H, $N_{piperidine}$-CH_2), 2.29 (br, 6H, $N_{piperidine}$-CH_2), 1.49 (1m, 24H, $9N_{piperidine}CH_2$-CH_2, $3N_{arom}CH_2$-CH_2), 1.06 (1m, 6H, $CH_{2piperidine}$) ppm; UV (aq. MES-KCl) λ max, nm (ε x 10^{-4}, $M^{-1}cm^{-1}$): 255 (2.6), 313 (4.8); HR-MS (ESI) m/z 763.5393 [$(M+H)^+$] (Calcd for $C_{51}H_{67}N_6$: 763.5427). Anal. found C 63.5% , H 8.3%, N 8.4%; (Calcd for [$C_{51}H_{66}N_6 \cdot 3HCl \cdot 5H_2O$]: C63.6 %, H8.3%, N 8.7%).

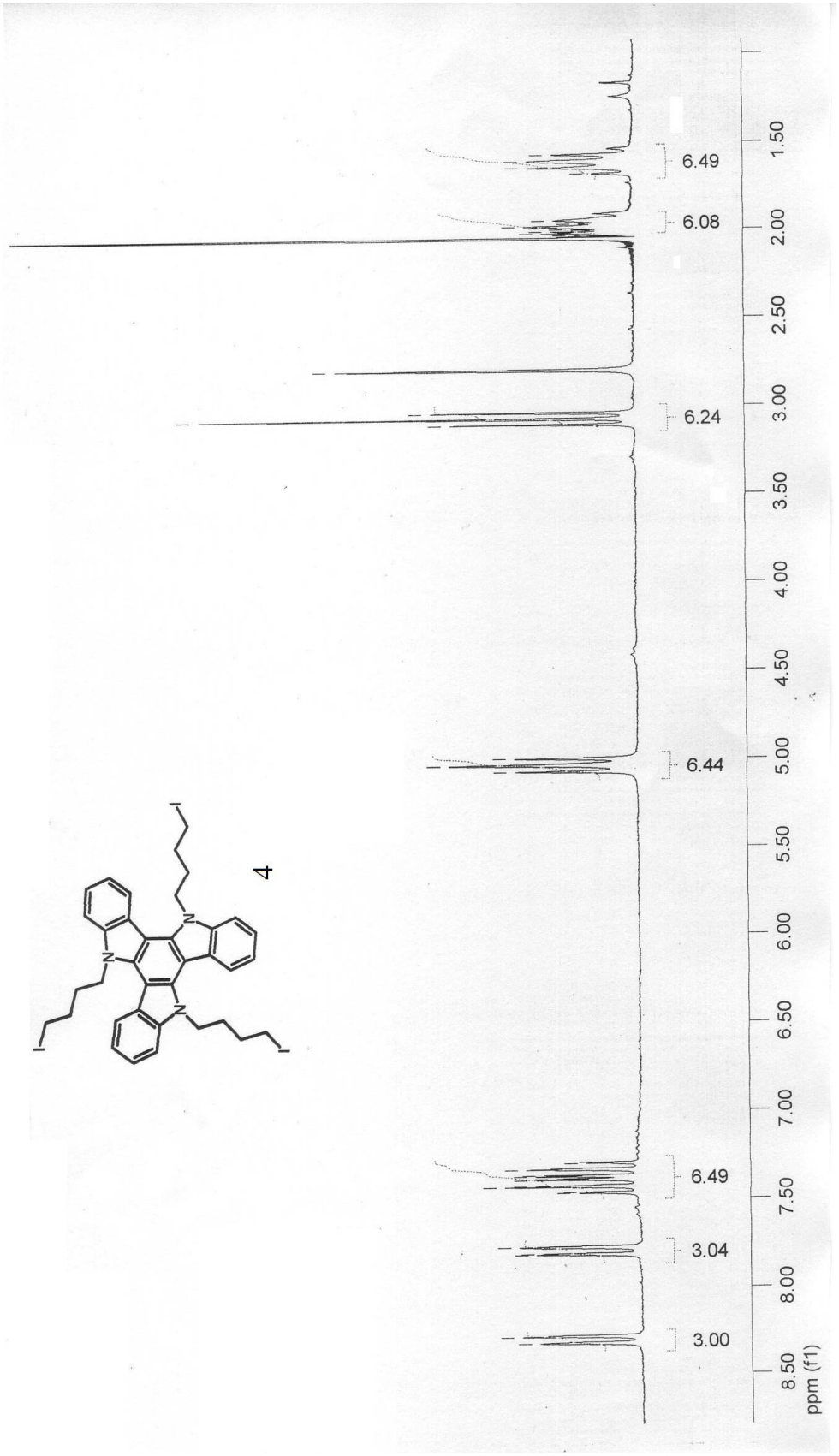
5,10,15-tris(4-iodobutyl)diindolo[3,2-a:3',2'-c]carbazole (4): ^{1}H NMR (200 MHz, acetone-d6) δ 8.30 (1d, 3H, J= 7.8, aromatic H), 7.79 (1d, 3H, J=7.8, aromatic H), 7.38 (1m, 6H, aromatic H), 5.03 (1t, 6H, J=7.4, N_{arom}-CH_2), 3.07 (1t, 6H, J= 7.0, I-CH_2), 1.89 (1 m, 6H, $N_{arom}CH_2$-CH_2), 1.61 (1m, 6H, ICH_2-CH_2) ppm; ^{13}C NMR (acetone-d6) δ: 141.4, 138.8, 123.5, 123.3, 121.8, 120.3, 111.3, 103.6 (8 aromatic C), 45.7, 30.6, 30.4, 6.1 (4 C) ppm, MS (ESI) m/z 892.43 [$(M+H)^+$] (Calcd for $C_{36}H_{37}I_3N_3$: 892.43).

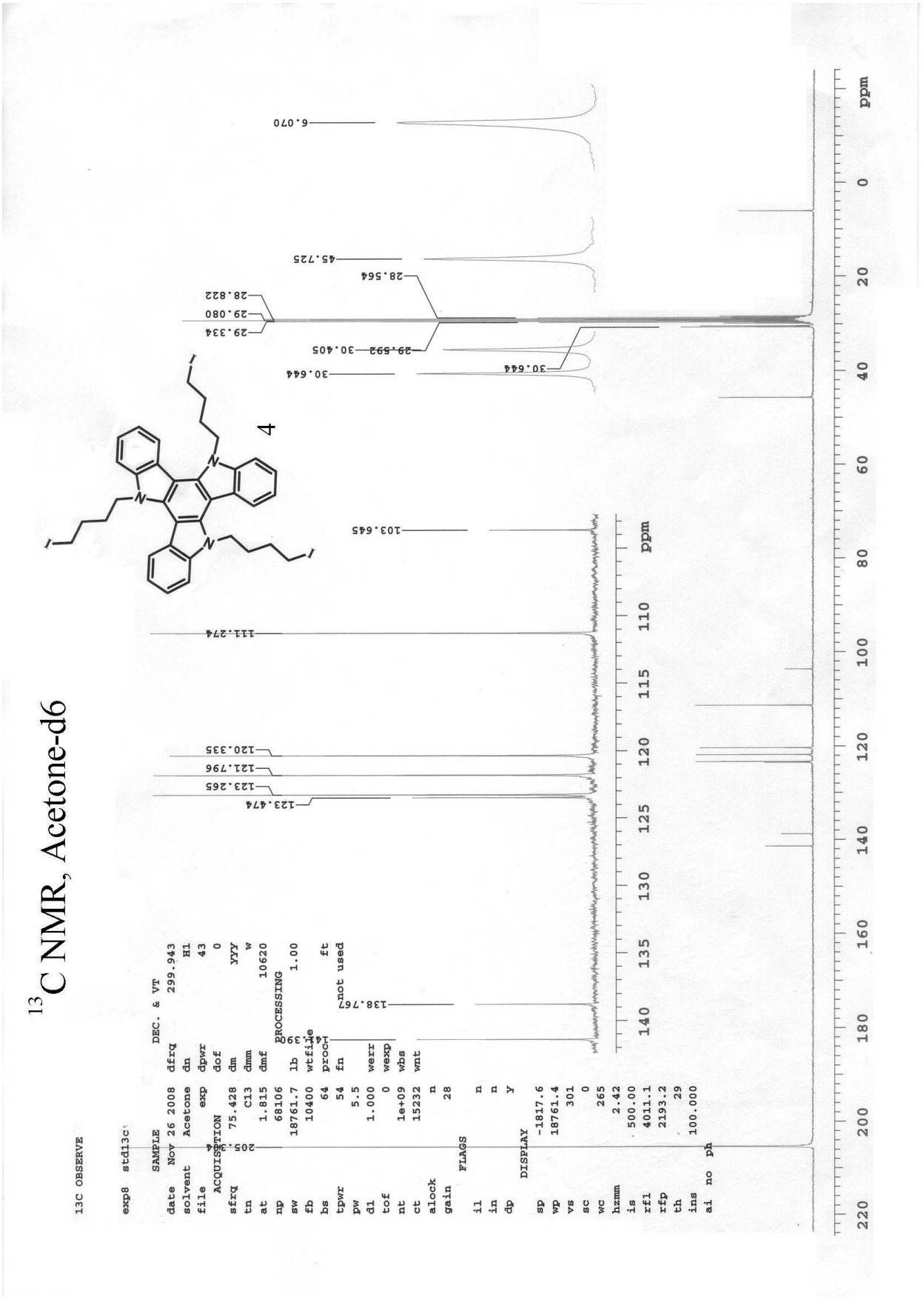
5,10,15-tris[4(1-piperidino)butyl]diindolo[3,2-a:3',2'-c]carbazole (3): ^{1}H NMR (200 MHz, chloroform-d) δ 8.26 (d, 3H, J=8.4, aromatic H), 7.68 (d, 3H, J=8.2, aromatic H), 7.39 (m, 6H, aromatic H), 4.95 (t, 6H, J=7.4, N_{arom}-CH_2), 2.25 (m, 18H, $N_{piperidine}$-CH_2), 1.99 (m, 6H, $N_{arom}CH_2$-CH_2), 1.51 (m, 18H, $N_{piperidine}CH_2$-CH_2), 1.39 (m, 6H, $CH_{2piperidine}$) ppm; ^{13}C NMR (chloroform-d) δ: 140.8, 138.6, 123.2, 122.7, 121.3, 119.7, 110.6, 103.0 (8 aromatic C), 58.2, 54.2, 46.5, 27.5, 25.5, 24.1, 23.4 ppm; UV (DMSO) λ max, nm (ε x10^{-4}, $M^{-1}cm^{-1}$): 267 (1.9), 318 (6.1), 354 (1.0).

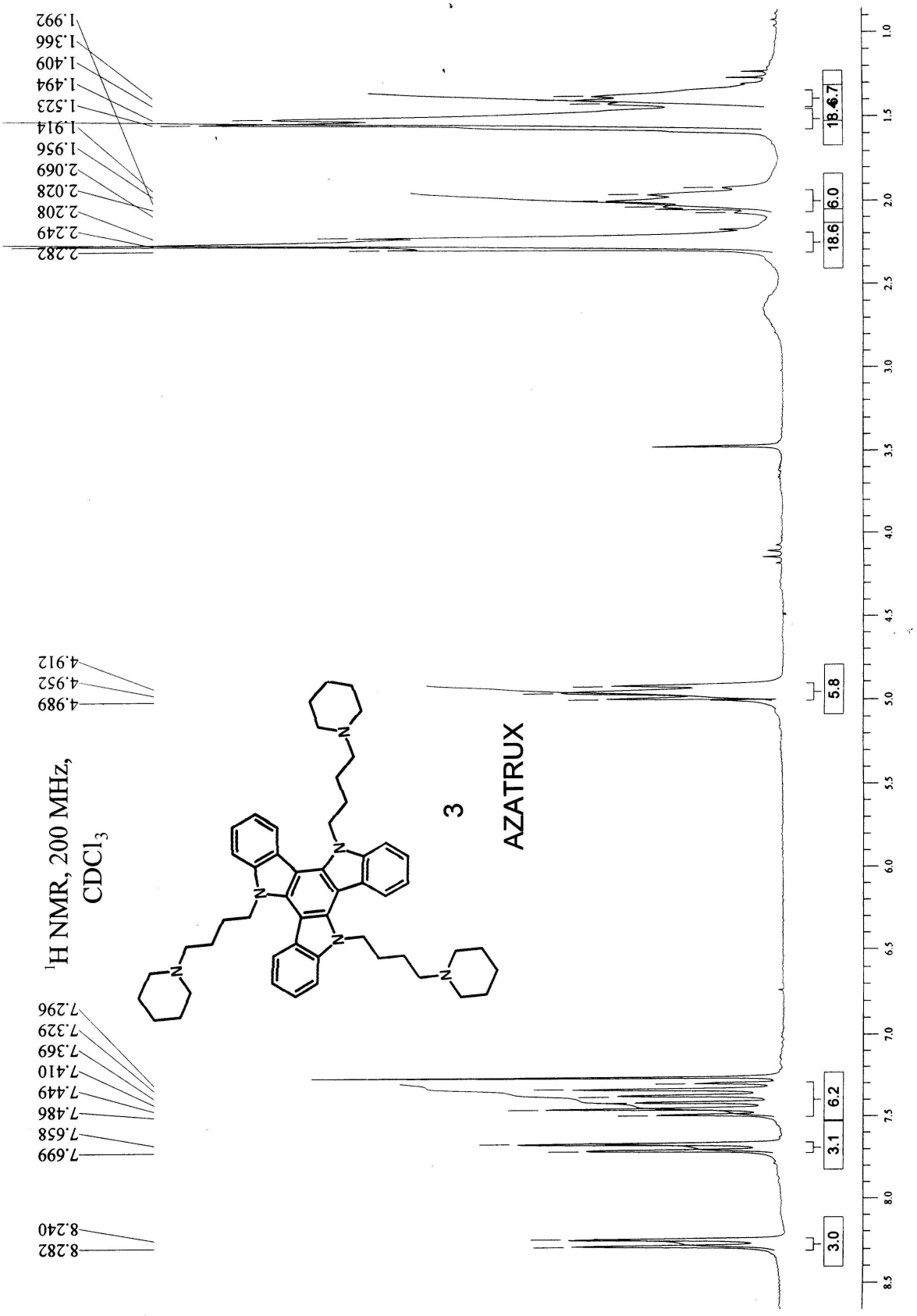

11.084
8.537
8.502
7.703
7.666
7.310
^{1}H NMR, 200 MHz,
Acetone-d6
HN
NH
NH
2
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
9.5
10.0
10.5
11.0
11.5
12.0

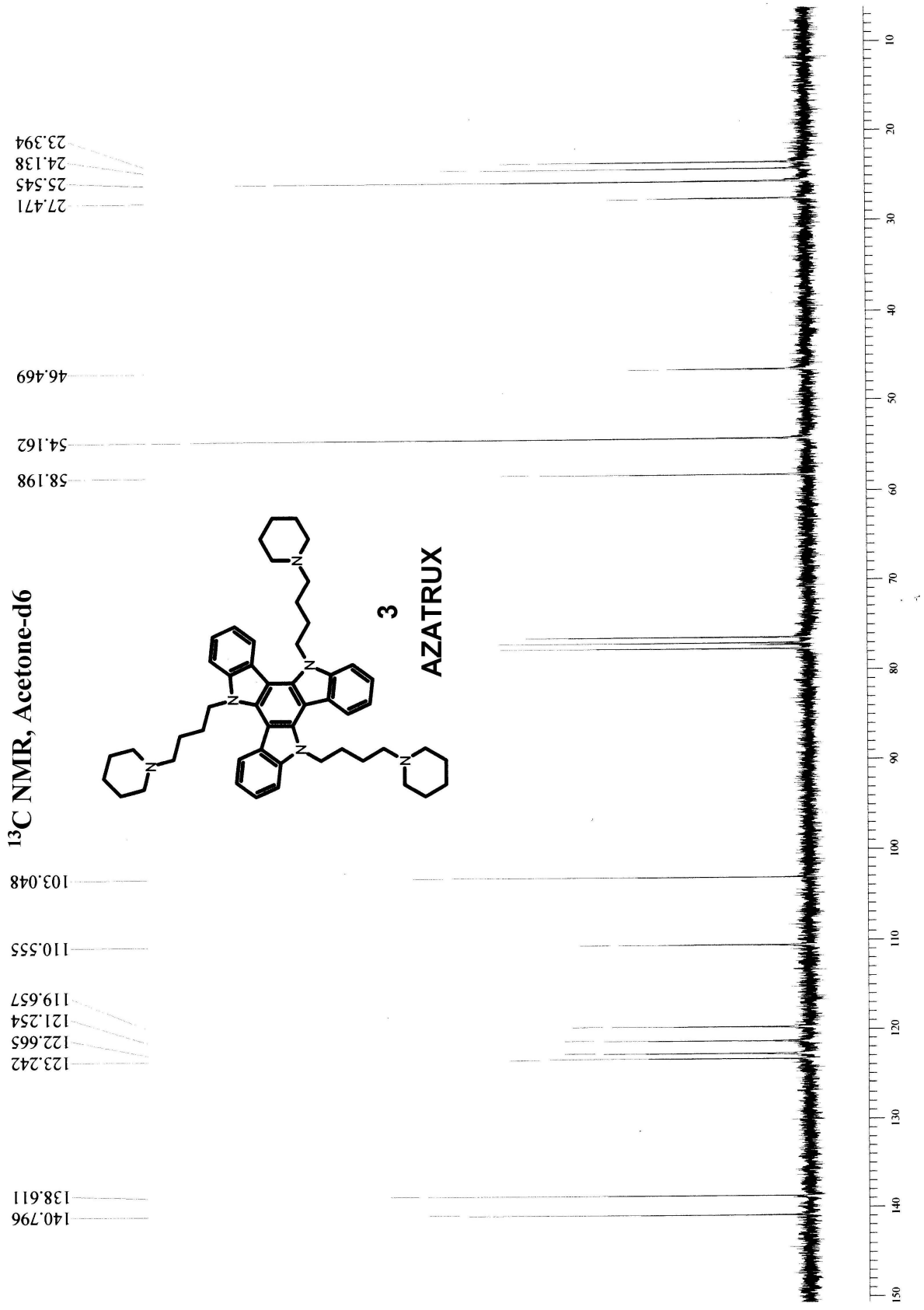

^{13}C NMR, ACETONE-d6
2
NH
HN
N
H
140.959
136.408
124.833
124.506
121.476
112.884
103.296
100
110
120
130
140
150
160

^{1}H NMR, 200 MHz, Acetone-d6




^{13}C NMR, Acetone-d6
4
6.070
45.725
28.564
28.822
29.080
29.334
29.592
30.405
30.644
103.645
111.274
120.335
121.796
123.265
123.474
138.767
ppm


1.992
2.366
2.409
2.494
2.523
2.914
2.956
3.069
3.028
3.208
3.249
3.282
4.912
4.952
4.989
7.296
7.329
7.369
7.410
7.449
7.486
7.658
7.699
8.240
8.282
^{1}H NMR, 200 MHz, $CDCl_3$
3
AZATRUX
3.0
3.1
6.2
5.8
18.6
6.0
18.6.7


^{13}C NMR, Acetone-d6
3
AZATRUX
23.394
24.138
25.545
27.471
46.469
54.162
58.198
103.048
110.555
119.657
121.254
122.665
123.242
138.611
140.796

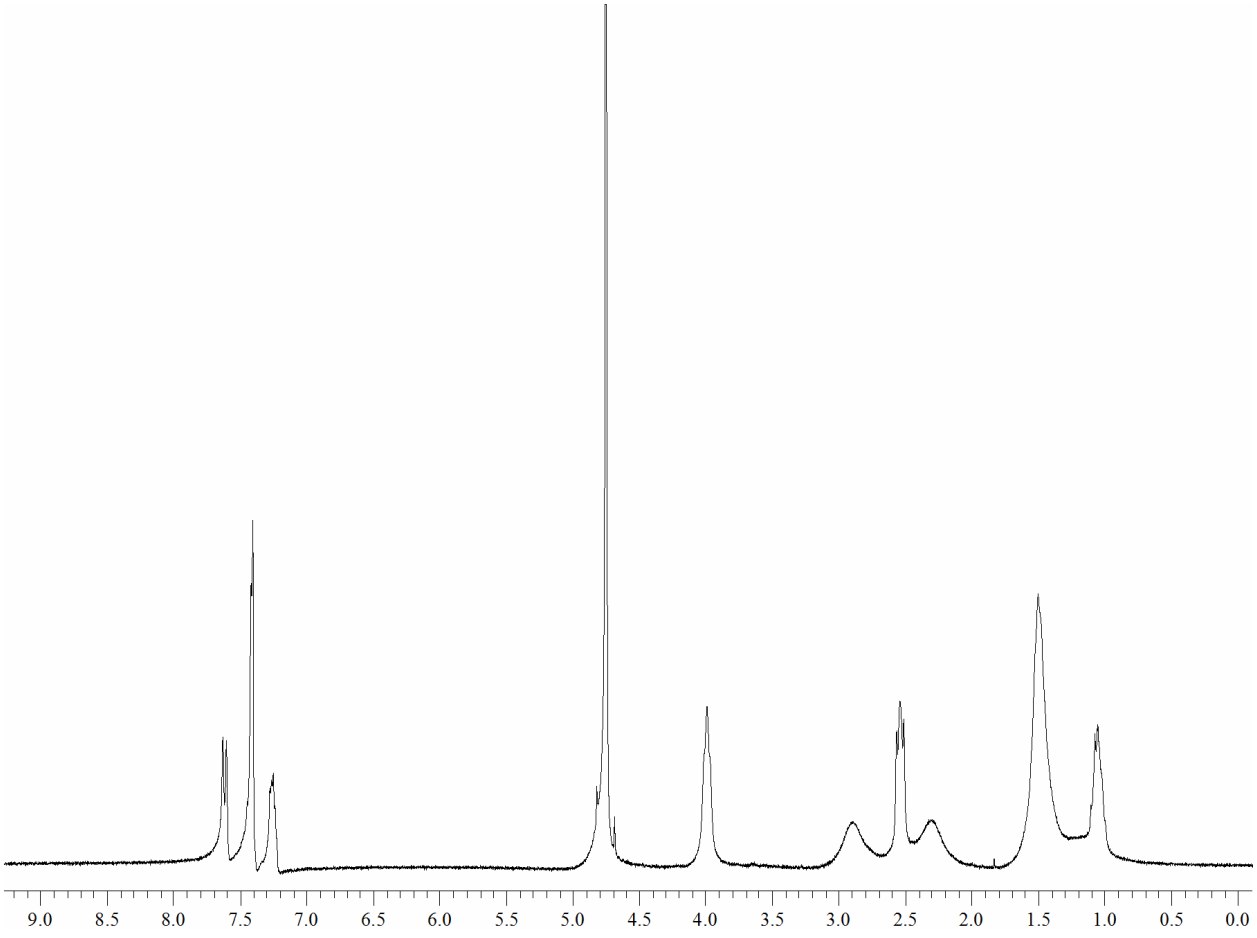


Figure S1: ^{1}H-NMR spectrum of AZATRUX as a hydrochloride in D_2O.

IV. Evaluation of the binding to quadruplex and duplex DNA by electrospray ionization mass spectrometry (ESI-MS):

ESI-MS spectra were recorded on a Micromass Q-TOF MICRO spectrometer (now Waters) in the negative ionization mode. The rate of sample infusion into the mass spectrometer was 10 µl/min and the capillary voltage was set to -2.6 kV. The source temperature was adjusted to 70°C, the cone voltage to 30 V and the collision energy to 5V. Full scan MS spectra were recorded in the m/z range between 800 and 2500, with 100 acquisitions per spectrum. Data were analyzed using the MassLynx software developed by Waters. Samples preparation (in 150 mM ammonium acetate buffer) and evaluation of the binding constants and percentage of bound DNA have been carried out according to the protocol described in a previous paper.[24]

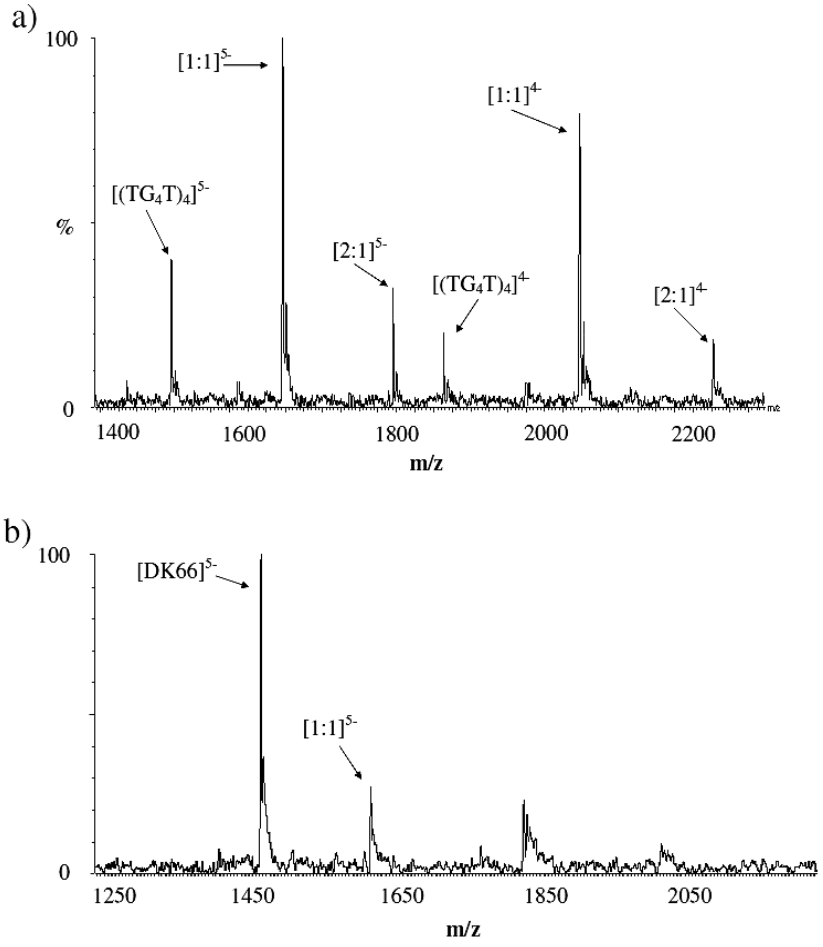


Figure S2: ESI mass spectra of $(TG_4T)_4$ in the presence of AZATRUX at 1:1 molar ratio (a) and of DK66 in the presence of AZATRUX at 1:4 molar ratio (b). Arrows indicate the peaks corresponding to the oligos alone and to the relative 1:1 or 2:1 drug/DNA complexes, in different charge states.

Competition experiments on F21T and $(TG_4T)_4$ oligos.

	[CT] = 0	**[Gq]:[CT] 1:1**	**[Gq]:[CT] 1:5**
F21T	46	39	14
$(TG_4T)_4$	75	64	44

Table S1: Values of the percentage of bound quadruplex DNA for samples containing a fixed amount of both drug and G-quadruplex DNA (5 μM, 1:1 ratio) and different amounts of calf thymus DNA (CT), at the indicated quadruplex/duplex ratios (in phosphate ions). All values should be considered with an error estimated on at least three independent experiments of about ±5%.

	[CT] = 0	**[Gq]:[CT] 1:1**	**[Gq]:[CT] 1:5**
F21T	1	0.85	0.30
$(TG_4T)_4$	1	0.85	0.59

Table S2: Values of normalized percentage bound quadruplex (N%) are elaborated starting from the percentages reported in Table S1 using the following equation:

$$N\% = \frac{\%\text{quadruplex bound in presence of CT}}{\%\text{quadruplex bound in absence of CT}}$$

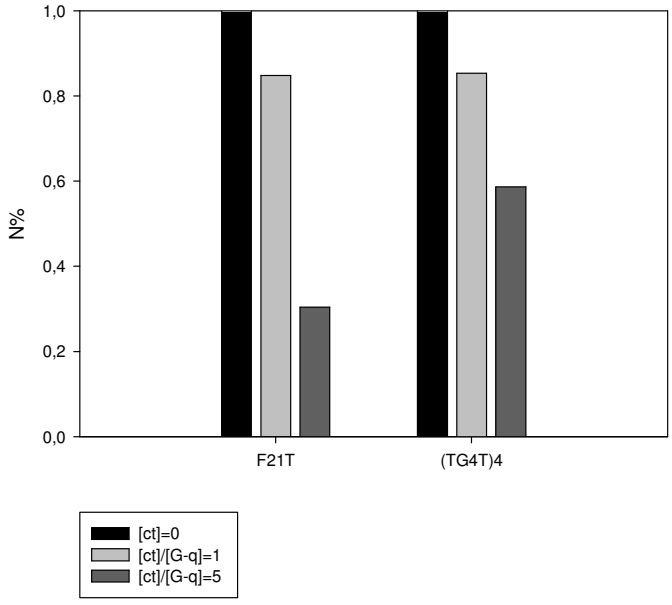


Figure S3: Competition experiments on F21T and $(TG_4T)_4$ oligos. Histogram diagrams reporting normalized percentage bound quadruplex (N%, Table S2).

V. Titrations by UV spectroscopy:

F21T was annealed in MES/KCl buffer, heating the sample at 90°C for ten minutes and cooling slowly to room temperature. Both F21T and sonicated CT DNA were diluted in MES/KCl buffer at an initial concentration of 200µM (R=20). Adding increasing volumes of 10µM drug stock the DNA/drug ratio (R) was decreased until a final DNA concentration of 1µM (R=0.1), while taking constant drug concentration. Two series of measures were performed for each DNA to confirm reproducibility.

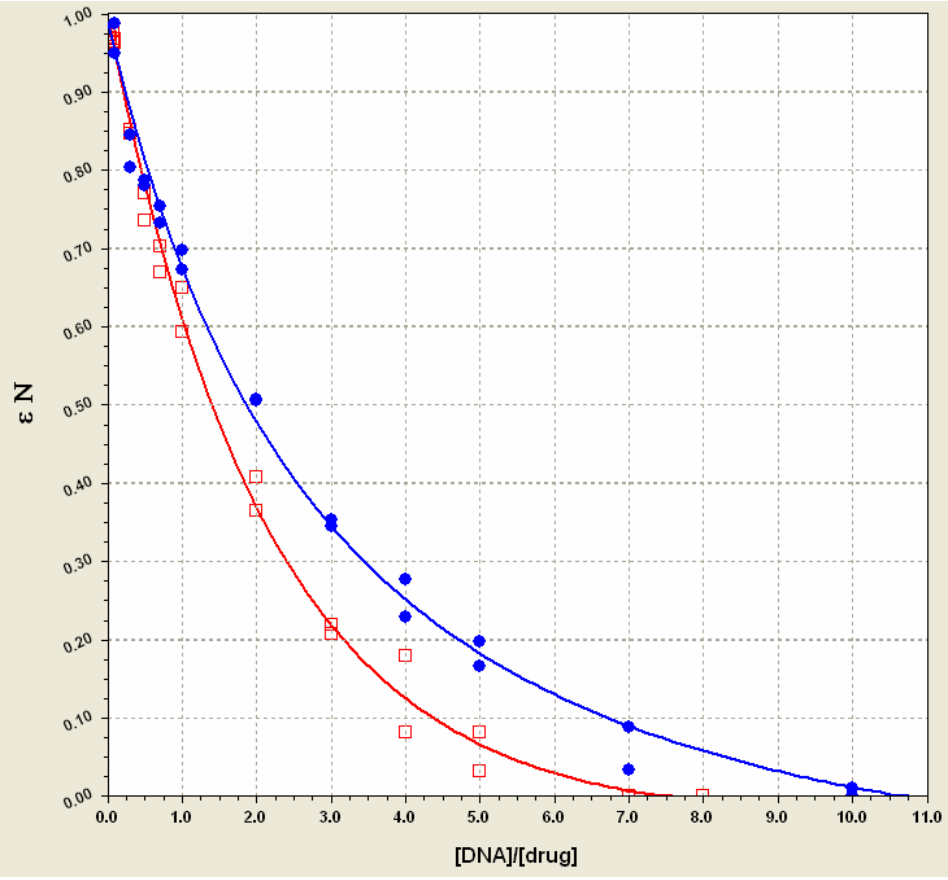


Figure S4: Normalized molar extinction coefficient (εN) versus DNA/drug concentration ratio of AZATRUX (10µM) at 313nm with F21T oligo (□, red) and CT DNA (●, blue).

VI. In vitro antitumor activity:

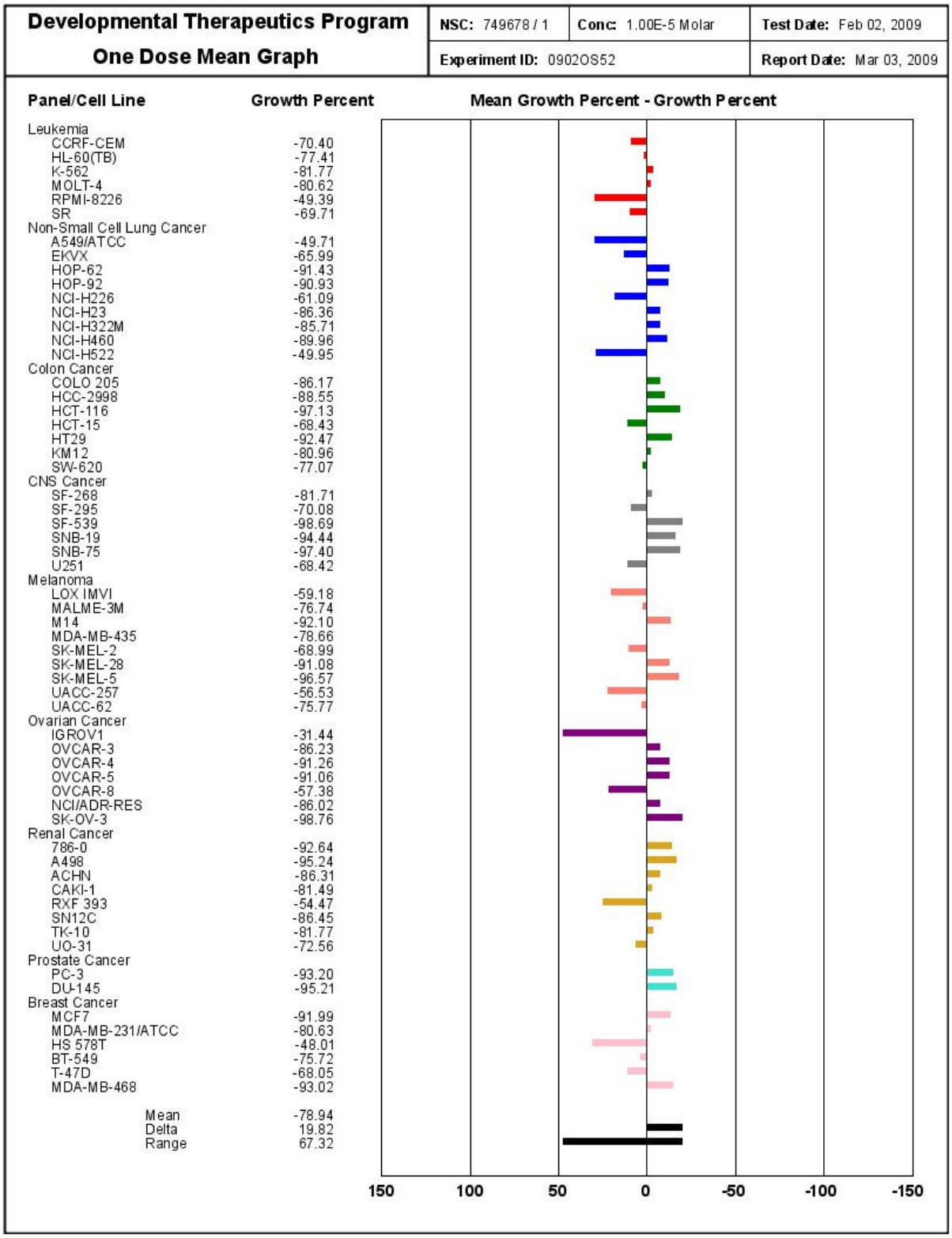


Figure S5: Growth percent of the indicated cell lines after 48 hours of incubation with 10μM AZATRUX solution, with respect to no-drug control, as reported by a preliminary study at the National Cancer Institute (NIH, Bethesda, MD, USA) over 60 cell lines.[29]