Supplementary Material for

Systematic study of SYBR Green chromophore reveals major improvement with one heteroatom difference

Ville K. Saarnio*, Johanna M. Alaranta and Tanja M. Lahtinen*

Department of Chemistry, Nanoscience Center, University of Jyväskylä, P.O. Box 35, 40014 Jyväskylä, Finland

CONTENTS

General information

Commercially available reagents and solvents were used as such without further purification apart from 1,2-dichloroethane (DCE) which was distilled over $CaCl_2$ and stored under 3 Å molecular sieves before use. K_2CO_3 was dried in an oven at 120 ˚C. ^1H and ^{13}C NMR spectra were measured with Bruker Avance III 500 MHz NMR spectrometer. Accurate HRMS spectra were measured with either Micromass LCT ESI-TOF or Agilent 6560 LC-IMMS-TOF mass spectrometer. 4-Methyl-1-phenylquinolin-2(1H)-one[1] **6**, methylmercapto-2-(methylthio)benzoxazolium tosylate[2] **4** and 2-(methylthio)benzothiazole[3] were prepared according to previously published procedures. 2-(methylthio)benzothiazole was N-methylated according to the previously reported synthesis.[2] The intermediate chloro-4-methyl-1-phenylquinolin-1-ium chloride **7** was synthesized in accordance to the previously reported procedure.[1]

Spectroscopy measurements were conducted always in room temperature in Hellma 110-QS (absorption) or 111-QS (fluorescence) cuvettes with 10 mm light path. Measurements in TE buffer and ctDNA solutions were conducted at pH 7.6. Calf thymus (ct) DNA was purchased from Sigma-Aldrich. Absorption spectra were recorded with Agilent Technologies Cary 8454 UV-vis spectrometer. All fluorescence spectra were collected with Varian Cary Eclipse Fluorescence spectrophotometer. For photobleaching experiments, a Nikon LHS-H100C-1 with halogen lamp was used as a light source. The light source power was measured using Thorlabs Model D3MM – thermopile device. Stock solutions of the synthesized dyes were prepared in dry dimethylsulfoxide in 19.6 mM concentration and stored in freezer.

Synthesis procedures

Synthesis of 2-((2-chloro-1-phenylquinolin-4(1H)-ylidene)methyl)-3-methylbenzo[d]oxazol-3-ium, OxCl (1)

4-Methyl-1-phenylquinolin-2(1H)-one[1] **6** (0.1030 g, 0.438 mmol) was dissolved in dry 1,2-DCE and $POCl_3$ (0.123 mL, 1.31 mmol) was added under nitrogen flow (Scheme S1). Light yellow solution was heated to 70 °C in an oil bath and stirred overnight in N_2 atmosphere. Solution was cooled to room temperature and solvent was removed. Resulting oil **7** was used in the next step without further purification.

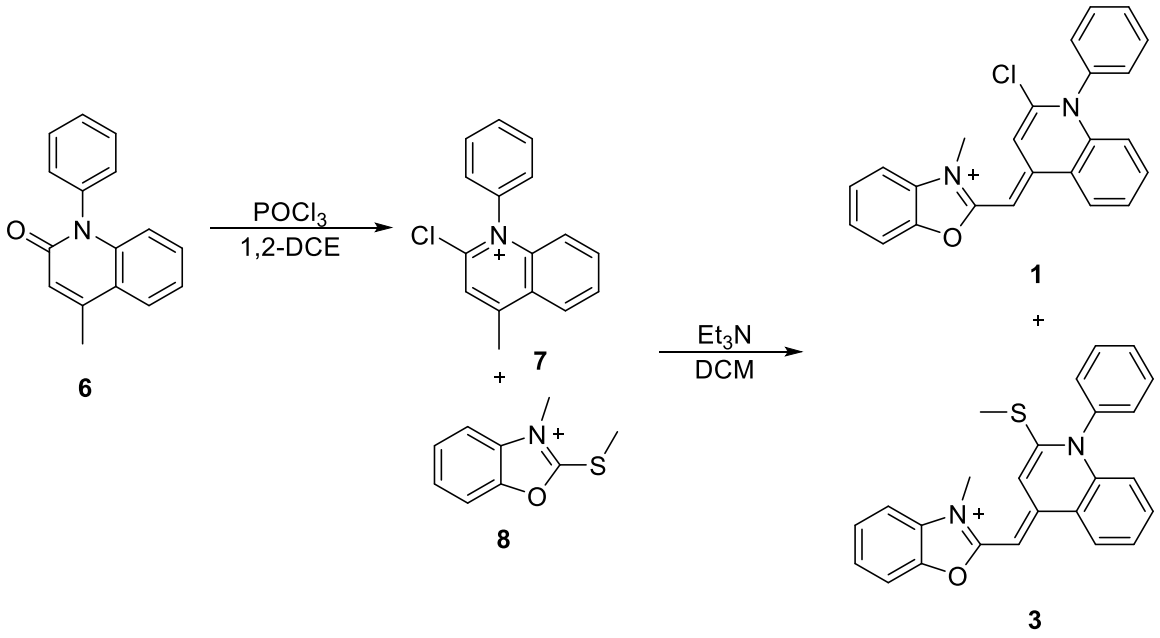
Oil **7** was dissolved in dry DCM under N_2 atmosphere in room temperature and **8**[2] (0.140 g, 0.438 mmol) was added. Triethylamine (97.6 µL, 0.7 mmol) was added to stirred solution. Reaction mixture was stirred at room temperature for 4 hours. Solvent was evaporated and product **1** and side product **3** were separated using flash chromatography on silica (0.040 - 0.063 mm). Mixture of MeOH and DCM was used (10% → 20%) as eluent. Yield for product OxCl **1** was 18.6 mg (10.1 %).

^1H NMR (500 MHz, MeOD) δ 8.65 (d, *J* = 7.8 Hz, 1H), 8.25 (s, 1H), 7.82 (d, *J* = 8.1 Hz, 1H), 7.76 (t, *J* = 2.5 Hz, 1H), 7.75 (t, *J* = 2.9 Hz, 2H), 7.71 (dd, *J* = 6.1, 4.7 Hz, 1H), 7.71 – 7.67 (m, 1H), 7.66 (d, *J* = 8.0 Hz, 1H), 7.57 (dd, *J* = 11.4, 4.1 Hz, 1H), 7.52 (d, *J* = 3.9 Hz, 2H), 7.51 (d, *J* = 2.3 Hz, 1H), 7.21 (d, *J* = 7.9 Hz, 1H), 7.03 – 6.99 (m, 1H), 6.43 (s, 1H), 3.97 (s, 3H).

^{13}C NMR (126 MHz, MeOD) δ 164.02, 152.58, 148.37, 145.67, 142.66, 141.73, 139.87, 134.81, 132.00, 129.96, 128.19, 127.95, 127.11, 126.88, 126.67, 123.58, 120.95, 112.49, 112.31, 110.81, 77.41, 31.39 ppm.

HRMS (ESI-TOF) m/z for $C_{24}H_{18}ClN_2O^+$: found 385.1107 $[M]^+$, required 385.1102.

3-methyl-2-((2-(methylthio)-1-phenylquinolin-4(1H)-ylidene)methyl)benzo[d]oxazol-3-ium, OxS **3**, has been previously characterized and reported.[4]



Scheme S1. Synthesis route for OxCl **1** and side product OxS **3**

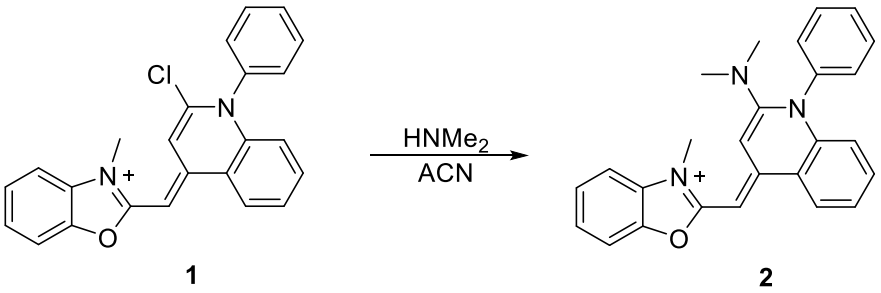
***Synthesis of 2-((2-(dimethylamino)-1-phenylquinolin-4(1H)-ylidene)methyl)-3-methylbenzo[d]oxazol-3-ium,* OxN (2)**

Product **1** was redissolved in ACN and $HNMe_2$ (5 mL, 39.5 mmol) was added (Scheme S2). Mixture was stirred at room temperature for 4 hours. Separation between water and DCM was done and organic layer was dried using $NaSO_4$. After evaporating the solvent, product was purified using flash chromatography on silica (0.040 - 0.063 mm). Mixture of MeOH and DCM was used (10% → 20%) as eluent. Yield for product OxN **2** was 10.3 mg (5.5 %).

^{1}H NMR (500 MHz, MeOD) δ 8.49 (d, *J* = 7.8 Hz, 1H), 7.81 (s, 1H), 7.73 (t, *J* = 7.5 Hz, 2H), 7.68 (t, *J* = 7.4 Hz, 2H), 7.63 (s, 1H), 7.62 (s, 1H), 7.55 (s, 1H), 7.54 (s, 1H), 7.46 (d, *J* = 5.2 Hz, 1H), 7.45 (d, *J* = 3.3 Hz, 1H), 7.33 (ddd, *J* = 8.5, 6.1, 2.7 Hz, 1H), 7.26 (d, *J* = 8.6 Hz, 1H), 5.99 (s, 1H), 3.80 (s, 3H), 2.93 (s, 6H).

^{13}C NMR (126 MHz, MeOD) δ 163.26, 159.96, 151.44, 148.08, 141.89, 140.55, 133.42, 133.23, 131.79, 131.29, 131.18, 127.14, 126.68, 126.28, 124.97, 122.72, 119.61, 111.50, 110.79, 102.76, 72.25, 43.35, 30.62 ppm.

HRMS (ESI-TOF) m/z for $C_{26}H_{24}N_3O^+$: found 394.1907 $[M]^+$, required 394.1914.



1 2

Scheme S2. Synthesis route for OxN **2**

***Synthesis of 3-methyl-2-((2-(methylthio)-1-phenylquinolin-4(1H)-ylidene)methyl)benzo[d]thiazol-3-ium,* ThzS (5)**

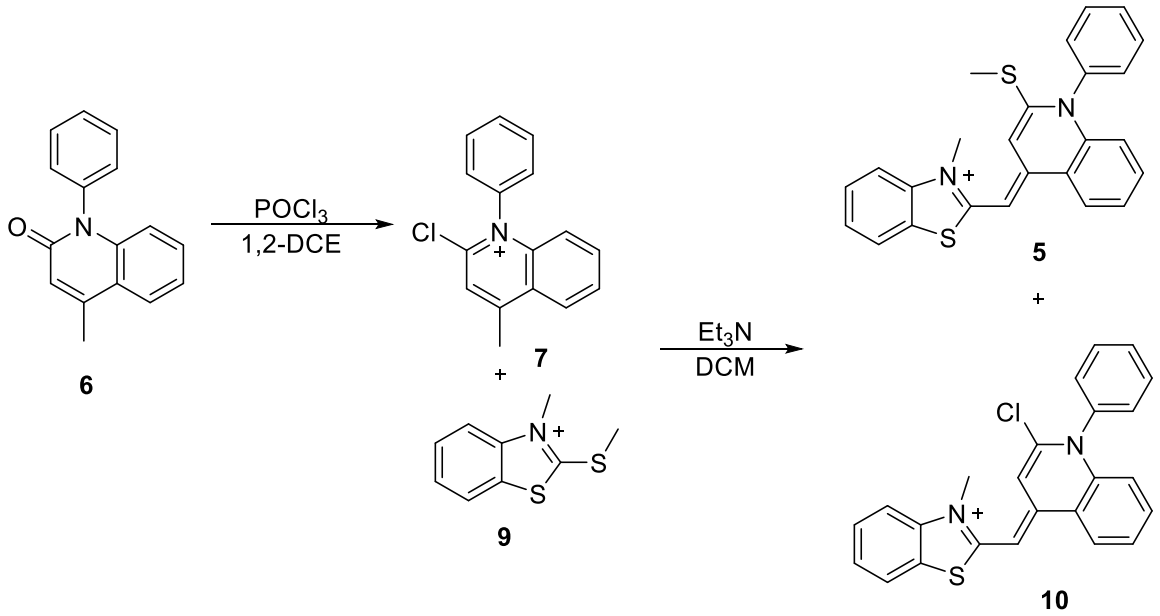
4-Methyl-1-phenylquinolin-2(1H)-one[1] **6** (0.1030 g, 0.438 mmol) was dissolved in dry 1,2-DCE and $POCl_3$ (0.123 mL, 1.31 mmol) was added under nitrogen flow (Scheme S3). Light yellow solution was heated to 70 °C in an oil bath and stirred overnight in N_2 atmosphere. Solution was cooled to room temperature and solvent was removed. Resulting oil **7** was used in the next step without further purification.

Oil **7** was dissolved in dry DCM under N_2 atmosphere in room temperature and **9** (0.1472 g, 0.438 mmol) was added. Triethylamine (97.6 µL, 0.7 mmol) was added to stirred solution. Reaction mixture was stirred at room temperature for 4 hours. Solvent was evaporated and products **5** and **10** were separated using flash chromatography on silica (0.040 - 0.063 mm). Mixture of MeOH and DCM was used (10% → 20%) as eluent. Yield for product ThzS **5** was 10.5 mg (5.3 %).

^{1}H NMR (500 MHz, MeOD) δ 8.65 (q, 1H), 8.03 (d, *J* = 7.9 Hz, 1H), 7.83 (s, 1H), 7.76 (d, *J* = 3.2 Hz, 1H), 7.75 (s, 1H), 7.71 – 7.70 (m, 1H), 7.69 (d, *J* = 3.3 Hz, 1H), 7.67 (s, 1H), 7.62 (d, *J* = 2.5 Hz, 1H), 7.55 (s, 1H), 7.53 (d, *J* = 3.4 Hz, 1H), 7.32 (t, *J* = 7.6 Hz, 1H), 7.13 (s, 1H), 7.08 (s, 1H), 6.89 (d, *J* = 6.6 Hz, 1H), 4.15 (s, 3H), 3.99 (d, *J* = 5.7 Hz, 3H).

^{13}C NMR (126 MHz, MeOD) δ 164.73, 153.80, 146.26, 142.52, 139.21, 134.66, 132.56, 132.02, 131.74, 130.05, 129.96, 128.35, 127.09, 126.31, 124.77, 124.22, 123.49, 118.16, 114.86, 111.12, 109.57, 91.09, 38.54, 34.91.

HRMS (ESI-TOF) m/z for $C_{25}H_{21}N_2S_2^+$: found 413.1136 $[M]^+$, required 413.1141.



Scheme S3. Synthesis route for product ThzS **5**.

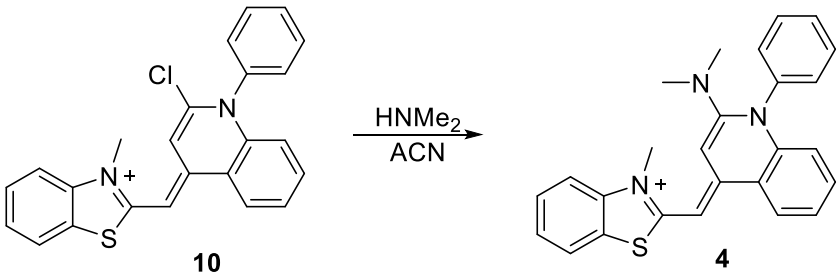
***Synthesis of 2-((2-(dimethylamino)-1-phenylquinolin-4(1H)-ylidene)methyl)-3-methylbenzo[d]thiazol-3-ium,* ThzN (4)**

Product **10** was redissolved in ACN and $HNMe_2$ (5 mL, 39.5 mmol) was added. The mixture was stirred at room temperature for 4 hours. Separation between water and DCM was done and organic layer was dried using $NaSO_4$. After evaporating the solvent, product was purified using flash chromatography on silica (0.040 - 0.063 mm). Mixture of MeOH and DCM was used (10% → 20%) as eluent. Yield for product ThzN **4** was 6.1 mg (3.1 %).

^{1}H NMR (500 MHz, MeOD) δ 8.51 (d, *J* = 8.2 Hz, 1H), 7.87 (t, *J* = 13.3 Hz, 1H), 7.79 – 7.71 (m, 2H), 7.68 (t, *J* = 7.4 Hz, 1H), 7.63 – 7.60 (m, 1H), 7.58 (s, 3H), 7.56 (s, 2H), 7.35 (ddd, *J* = 8.1, 5.6, 2.7 Hz, 1H), 7.30 (d, *J* = 8.5 Hz, 1H), 6.94 (s, 1H), 6.76 (s, 1H), 3.94 (s, 3H), 2.99 (s, 6H).

^{13}C NMR (126 MHz, MeOD) δ 160.07, 150.77, 142.38, 141.85, 140.53, 133.34, 131.85, 131.39, 131.19, 129.37, 126.81, 126.01, 125.36, 125.19, 123.73, 123.29, 119.66, 113.01, 102.73, 87.64, 43.52, 33.80 ppm.

HRMS (ESI-TOF) m/z for $C_{26}H_{24}N_3S^+$: found 410.1680 $[M]^+$, required 410.1686.



Scheme S4. Synthesis route for product ThzN **4**.

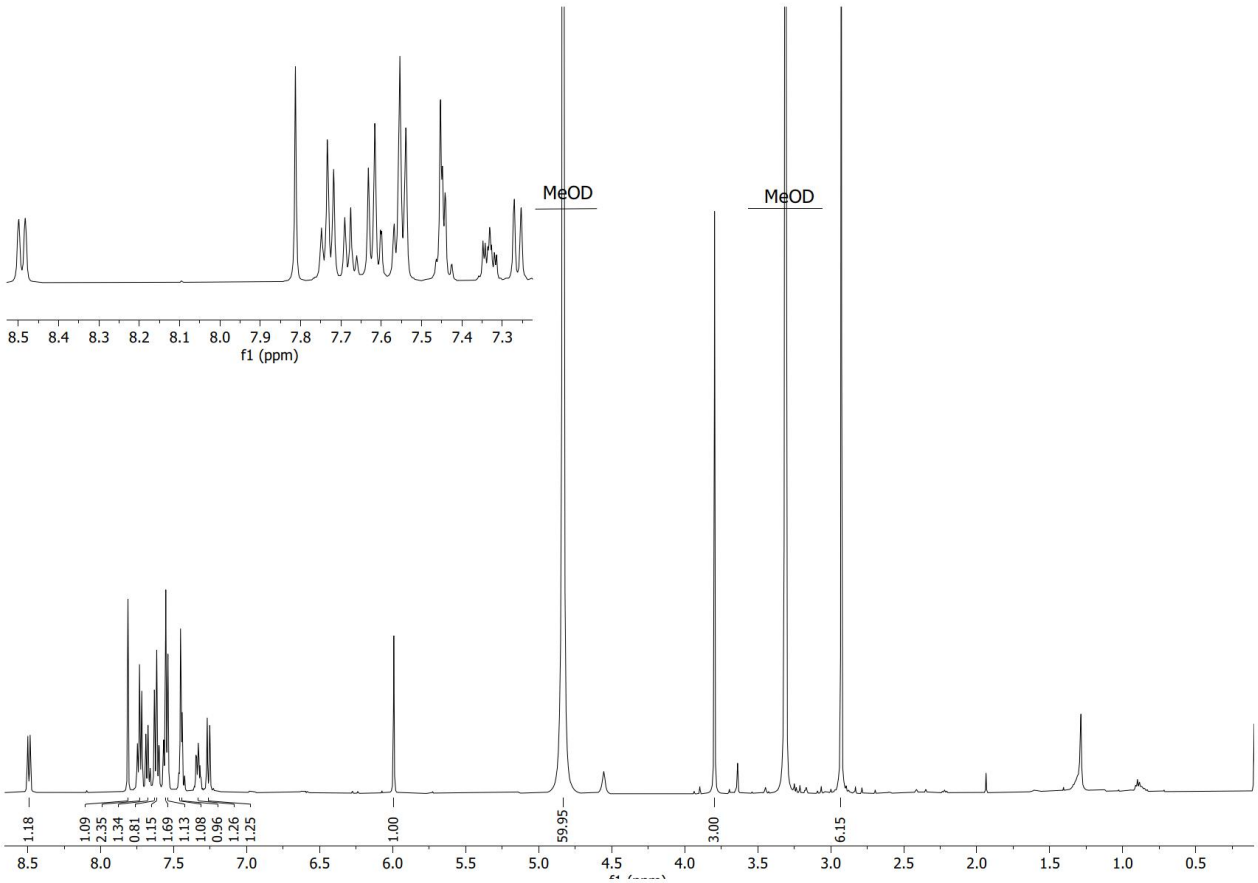


Fig. S1. ^{1}H NMR spectra of OxN **2** in MeOD.

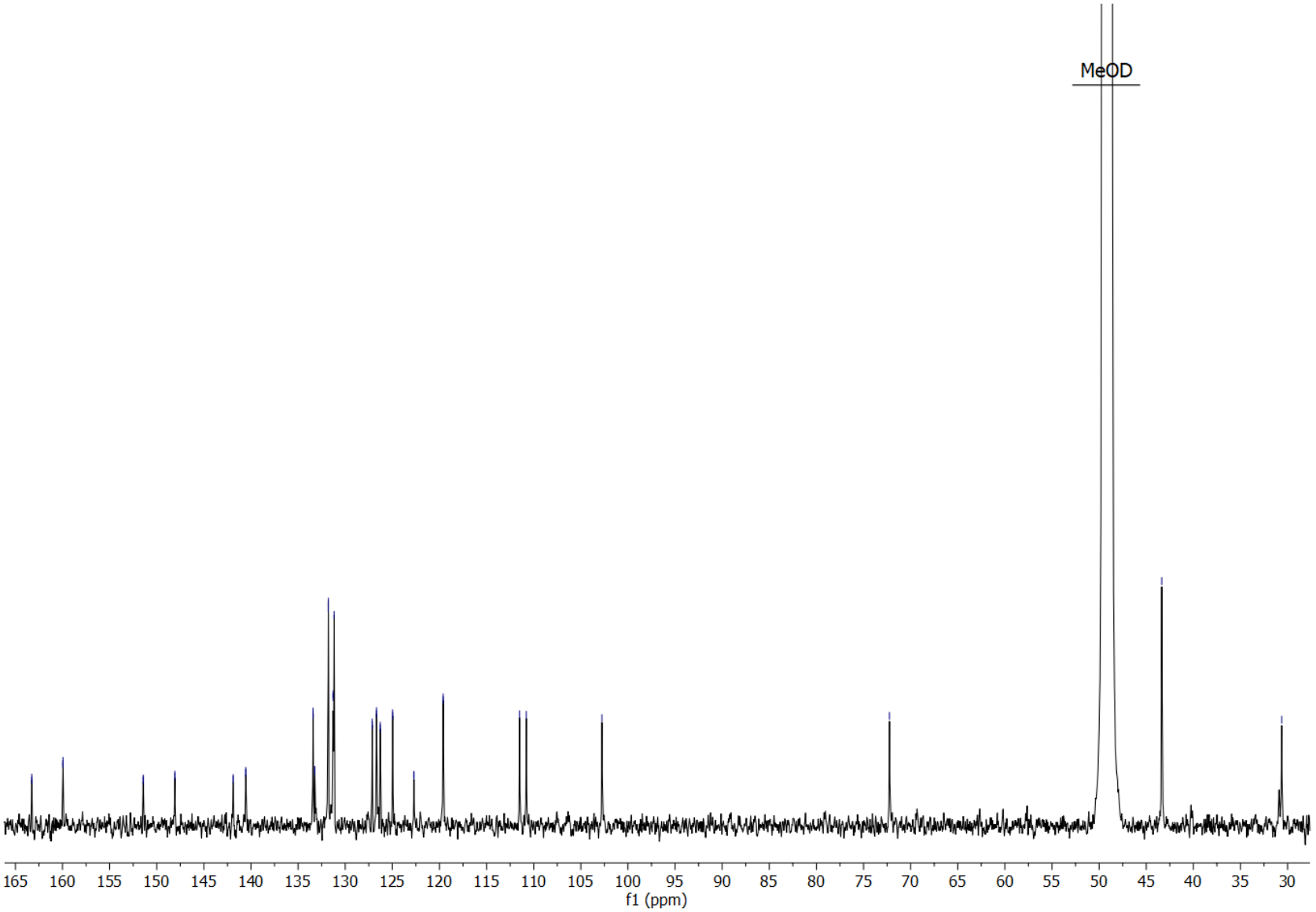


Fig. S2. ^{13}C NMR spectra of OxN **2** in MeOD

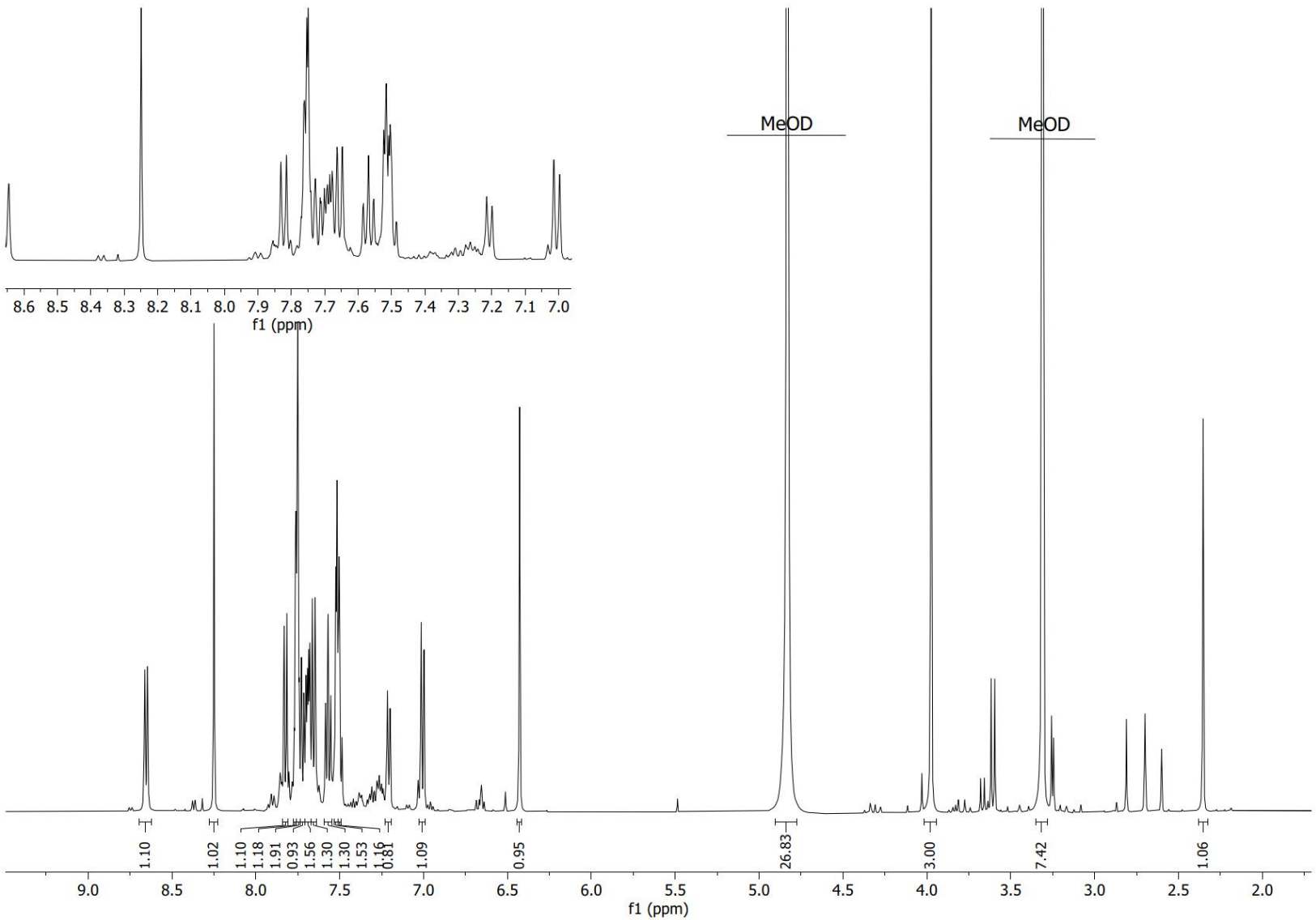


Fig. S3. ^{1}H NMR spectra of OxCl **1** in MeOD.

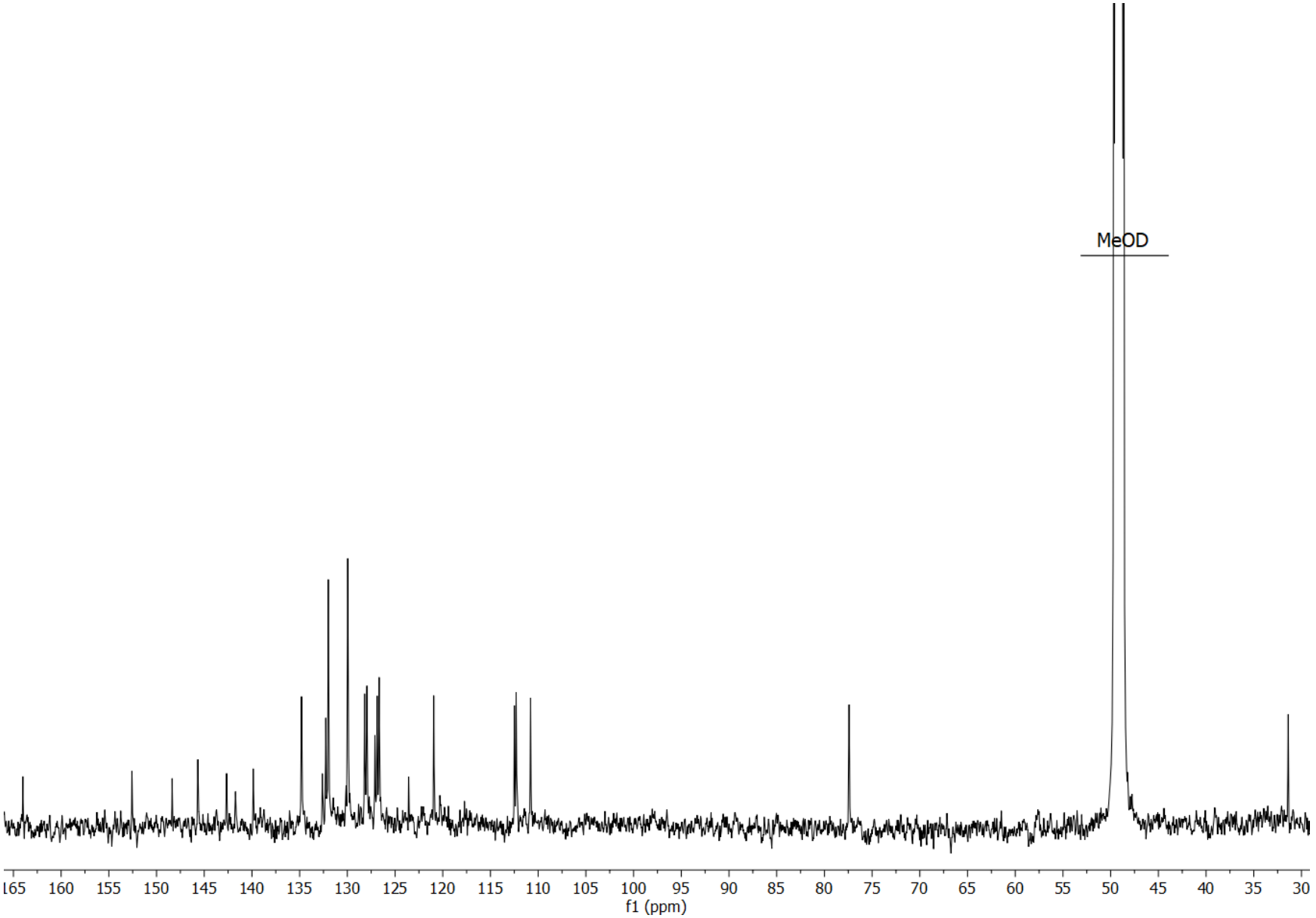


Fig. S4. ^{13}C NMR spectra of OxCl **1** in MeOD

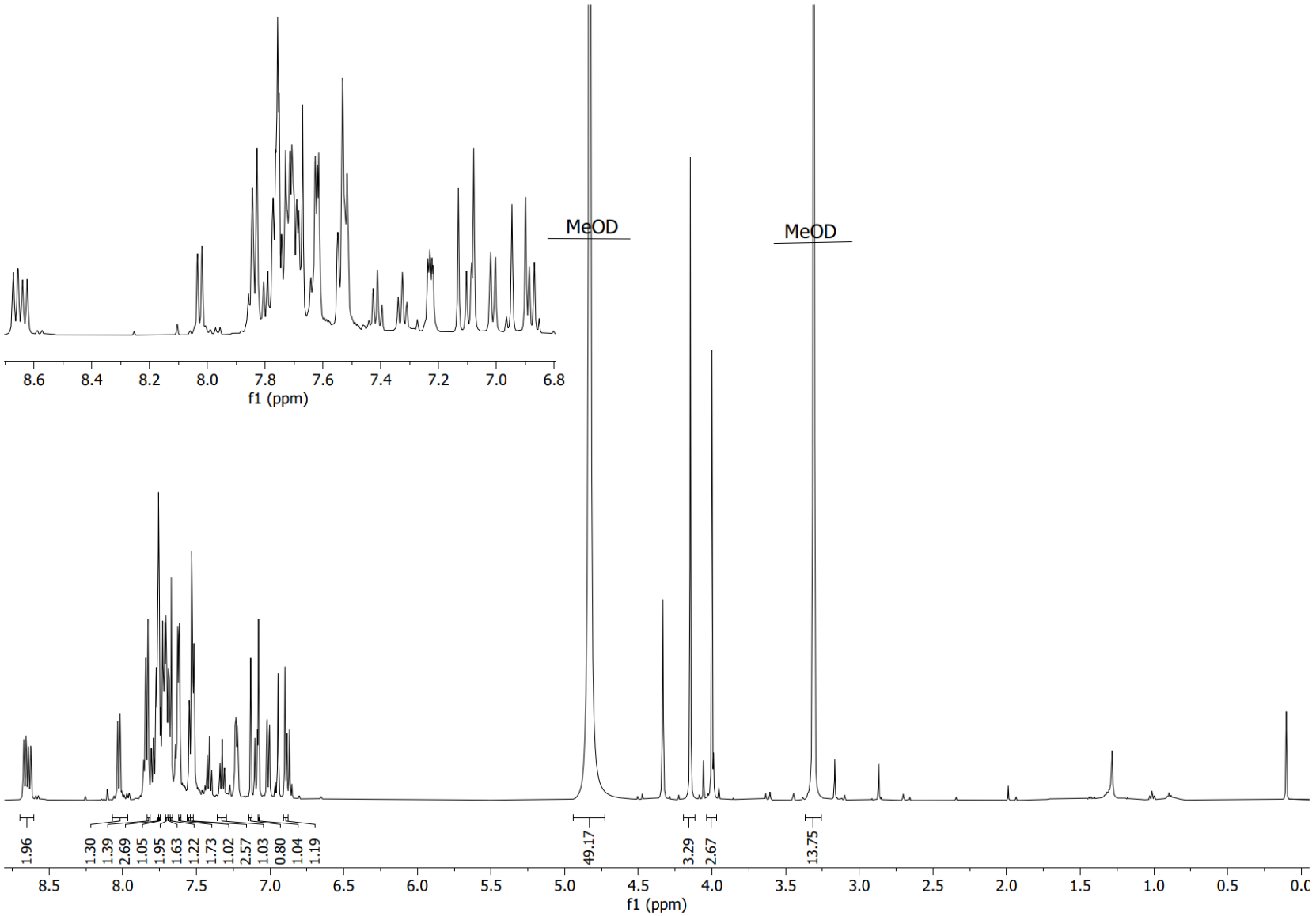


Fig. S5. ^{1}H NMR spectra of ThzS **5** in MeOD.

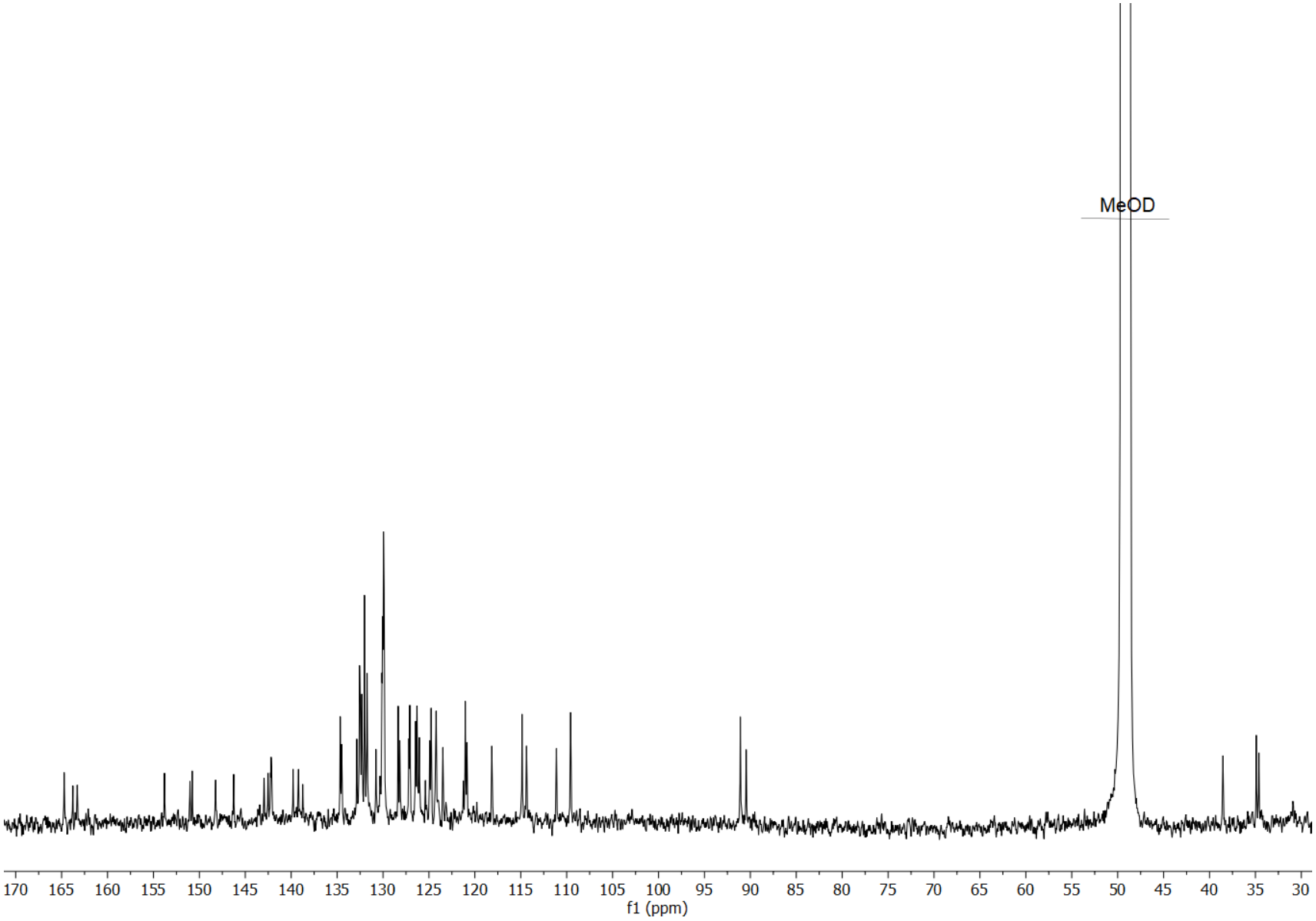


Fig. S6. ^{13}C NMR spectra of ThzS **5** in MeOD.

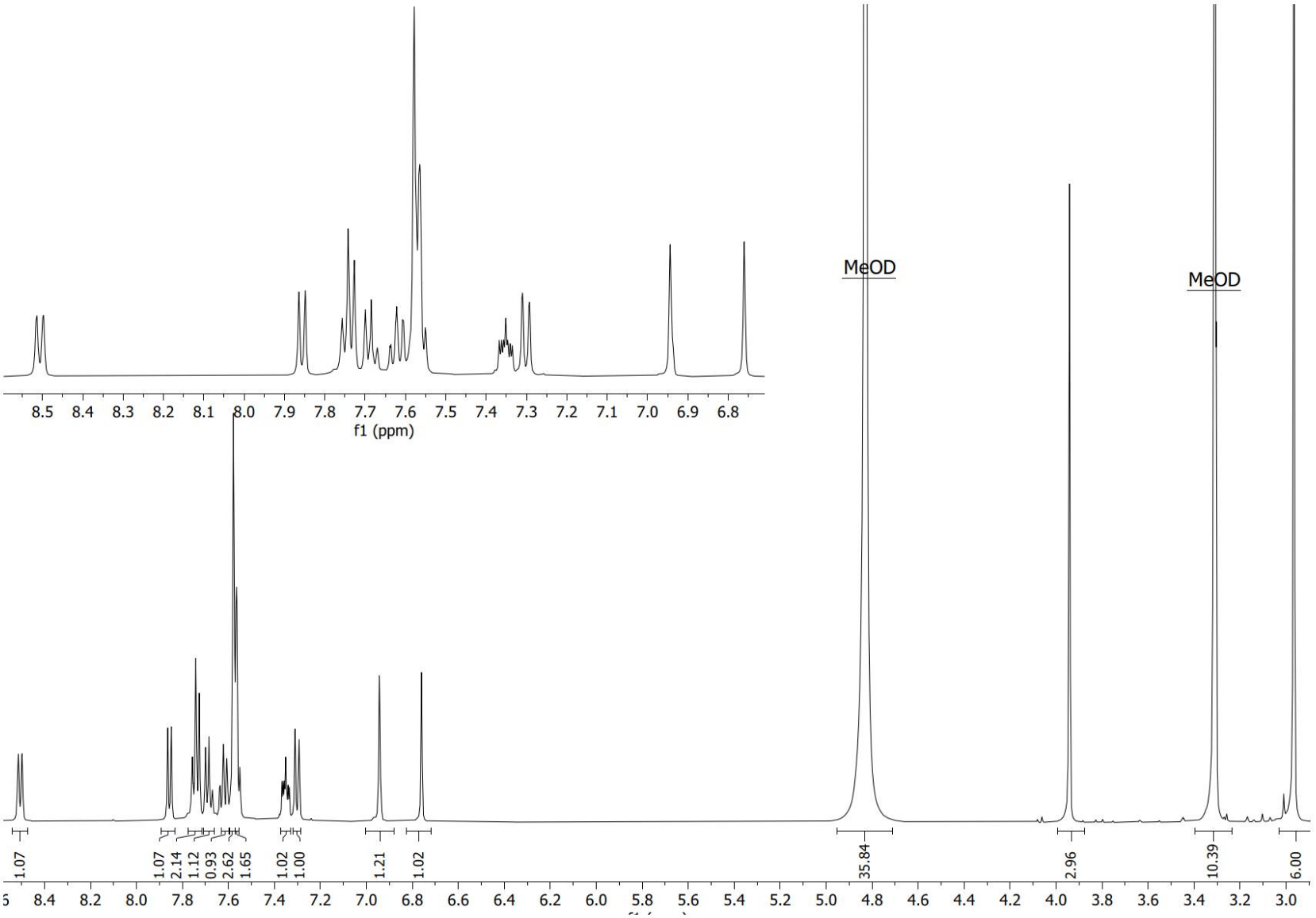


Fig. S7. ^{1}H NMR spectra of ThzN **4** in MeOD.

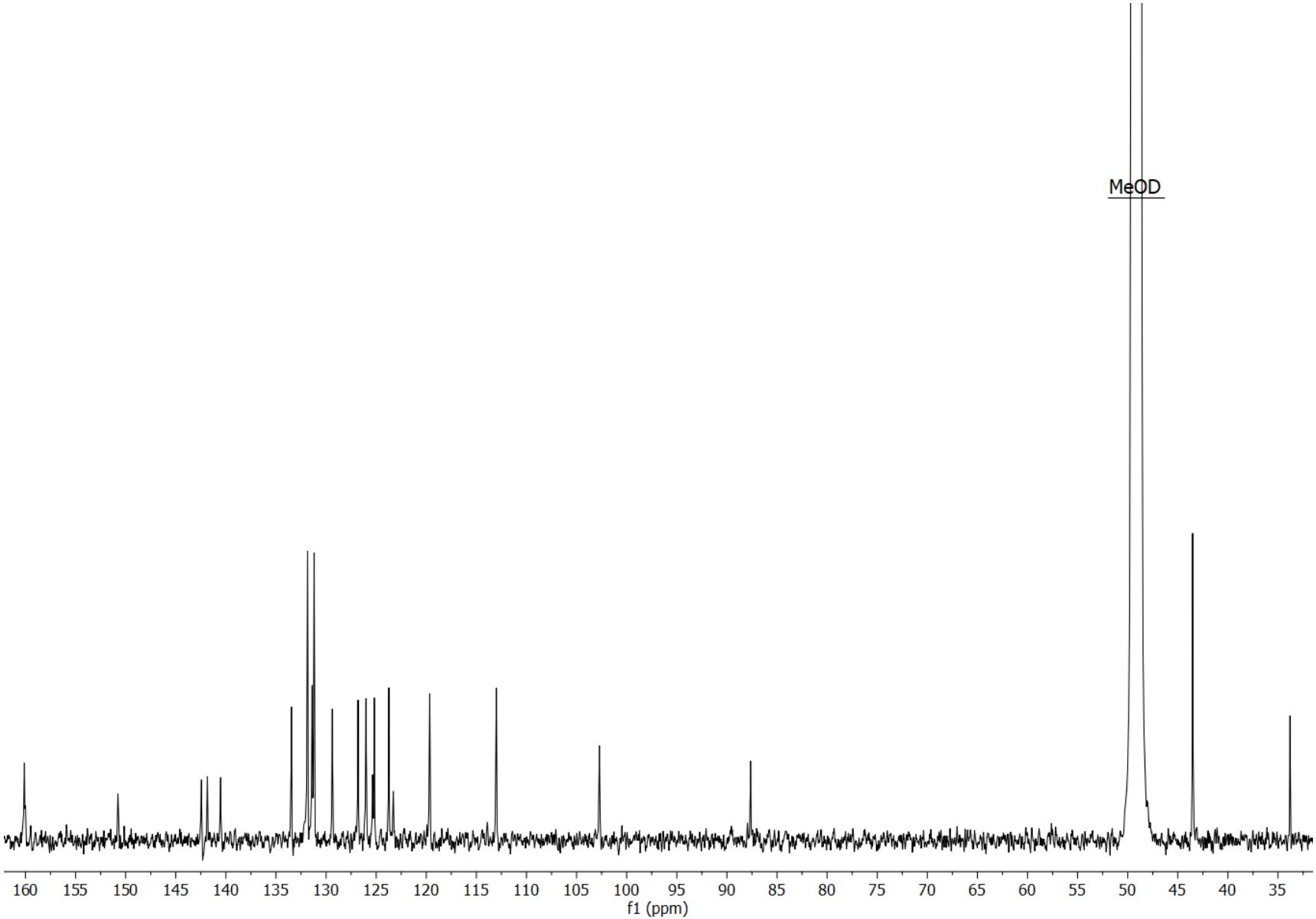


Fig. S8. ^{13}C NMR spectra of ThzN **4** in MeOD.

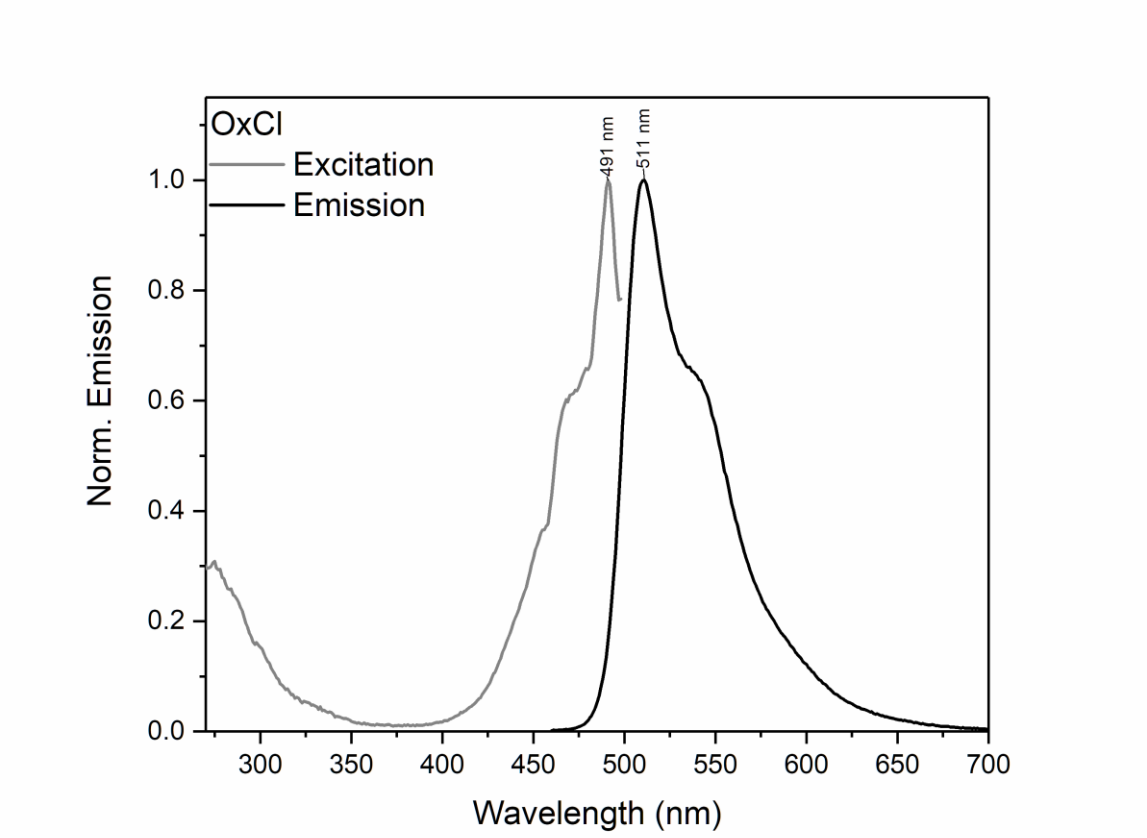


Fig. S9. Excitation and emission spectra of the OxCl **1** dye in binding with ctDNA in TE buffer.

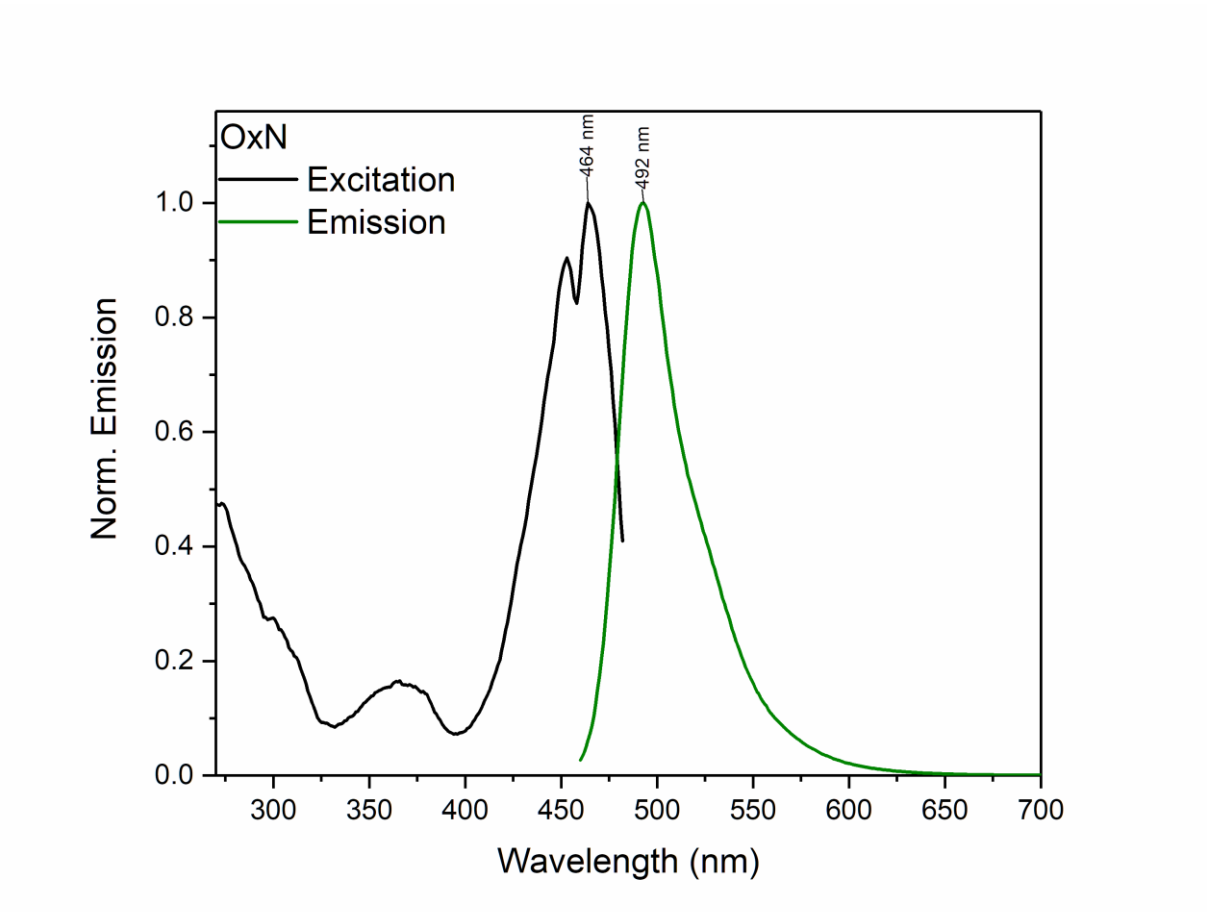


Fig. S10. Excitation and emission spectra of the OxN **2** dye in binding with ctDNA in TE buffer.

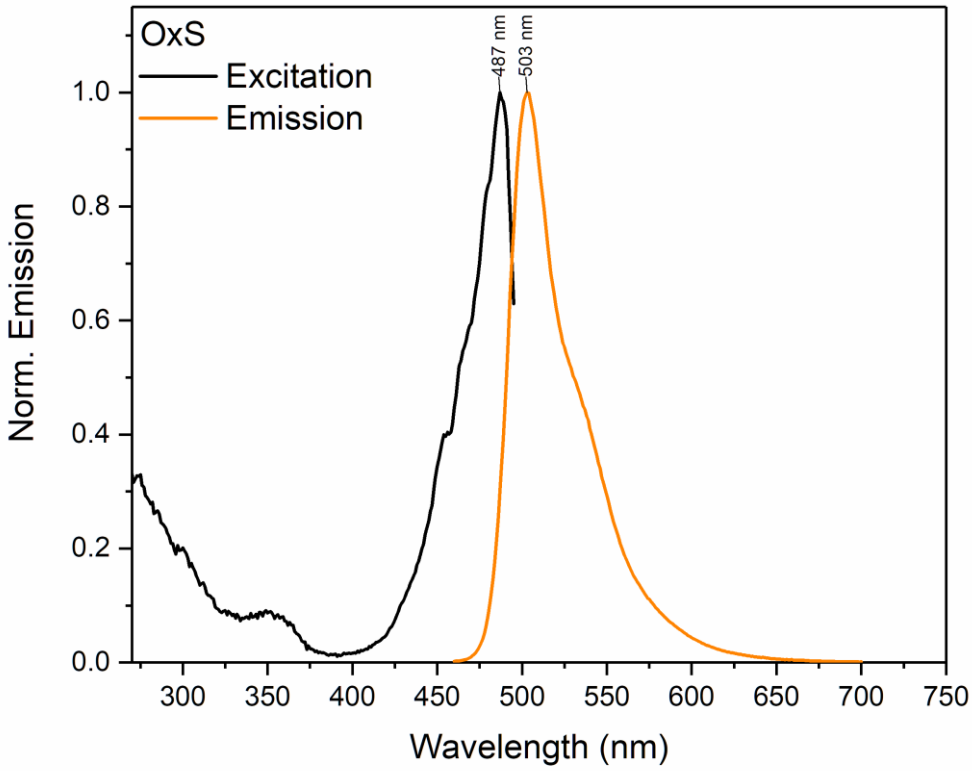


Fig. S11. Excitation and emission spectra of the OxS **3** dye in binding with ctDNA in TE buffer.

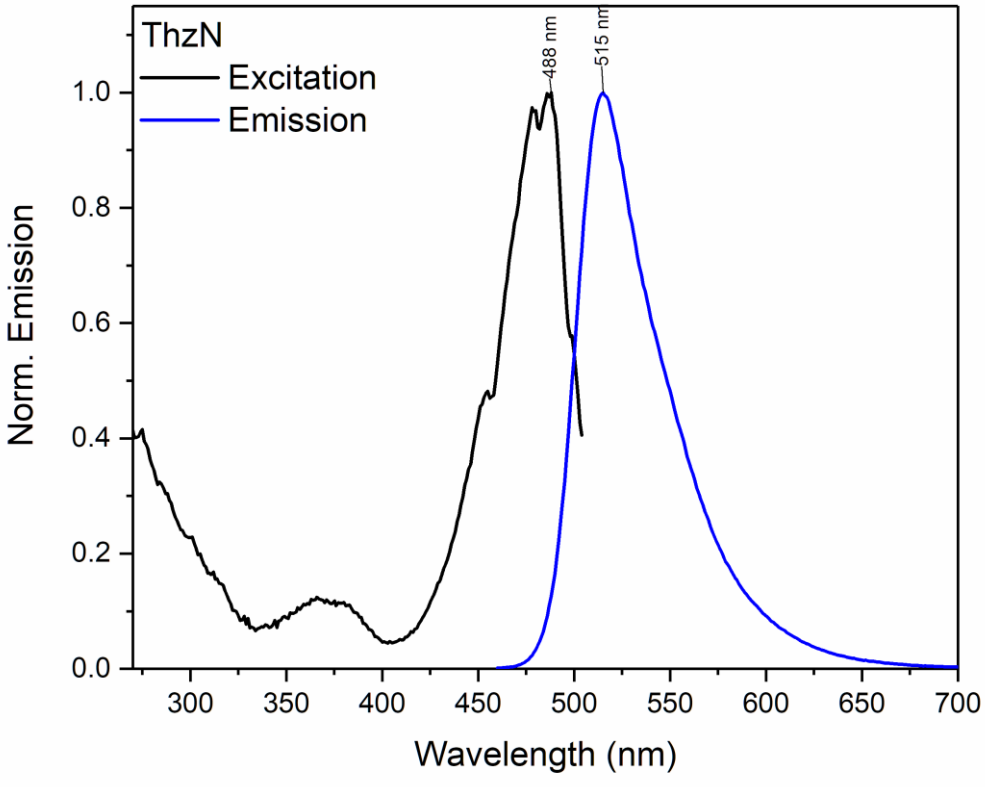


Fig. S12. Excitation and emission spectra of the ThzN **4** dye in binding with ctDNA in TE buffer.

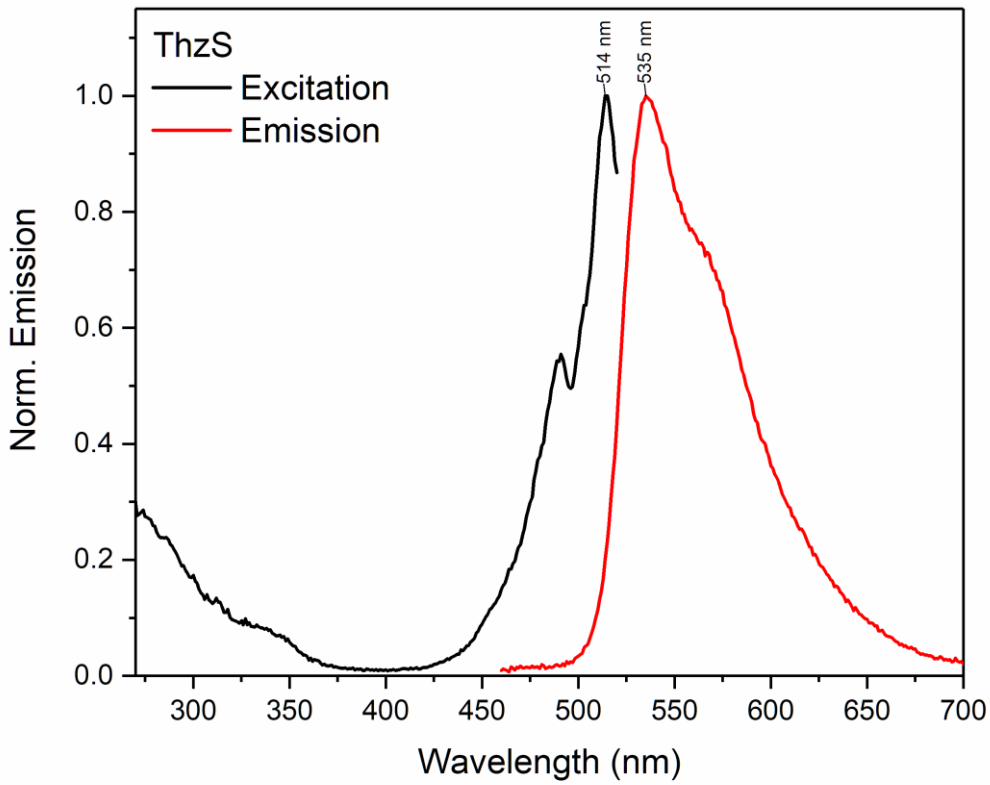


Fig. S13. Excitation and emission spectra of the ThzS **5** dye in binding with ctDNA in TE buffer.

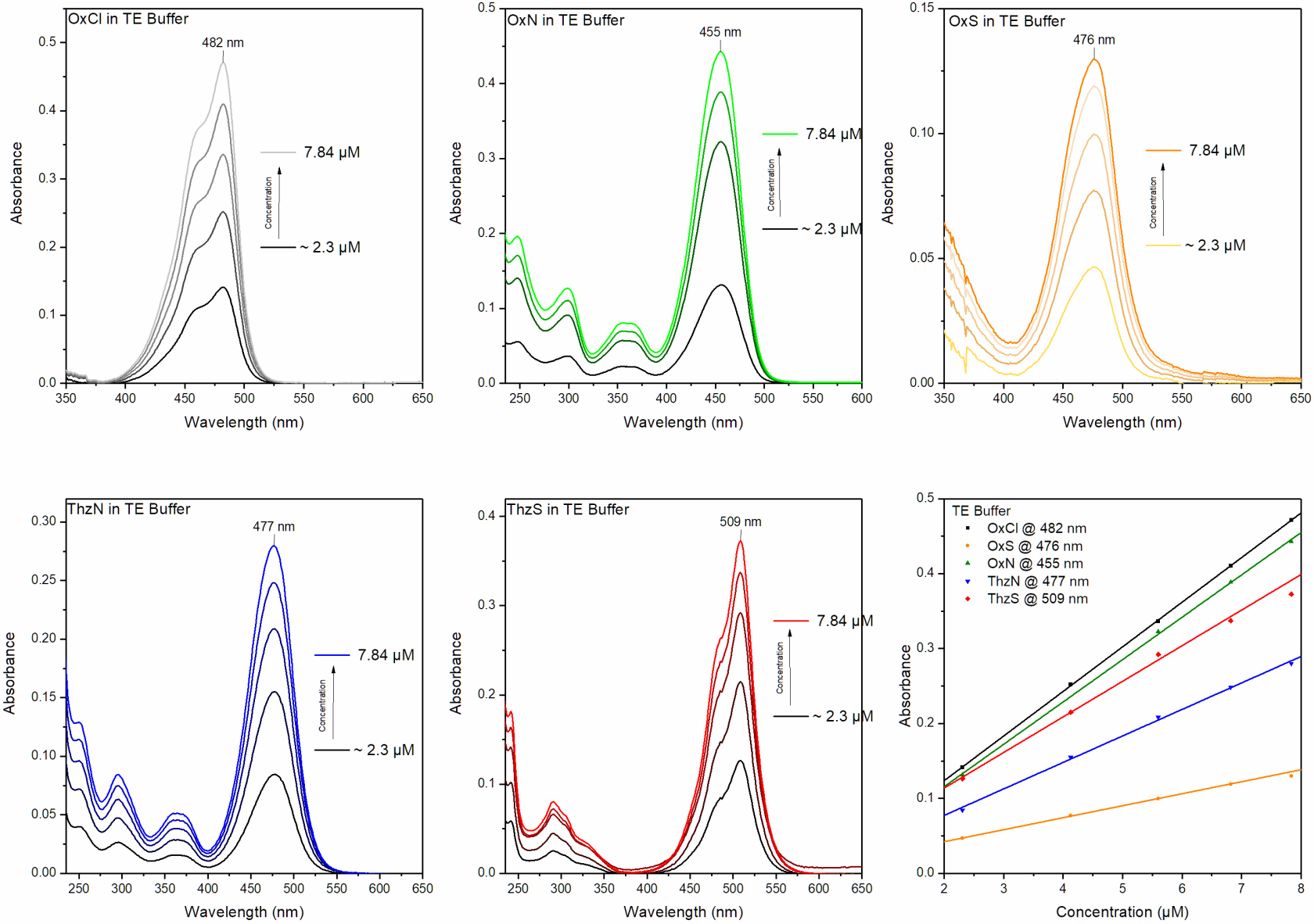


Fig. S14. UV-vis spectra of the synthesized dyes in five different concentrations in TE Buffer and linear fittings of the lowest energy maxima values.

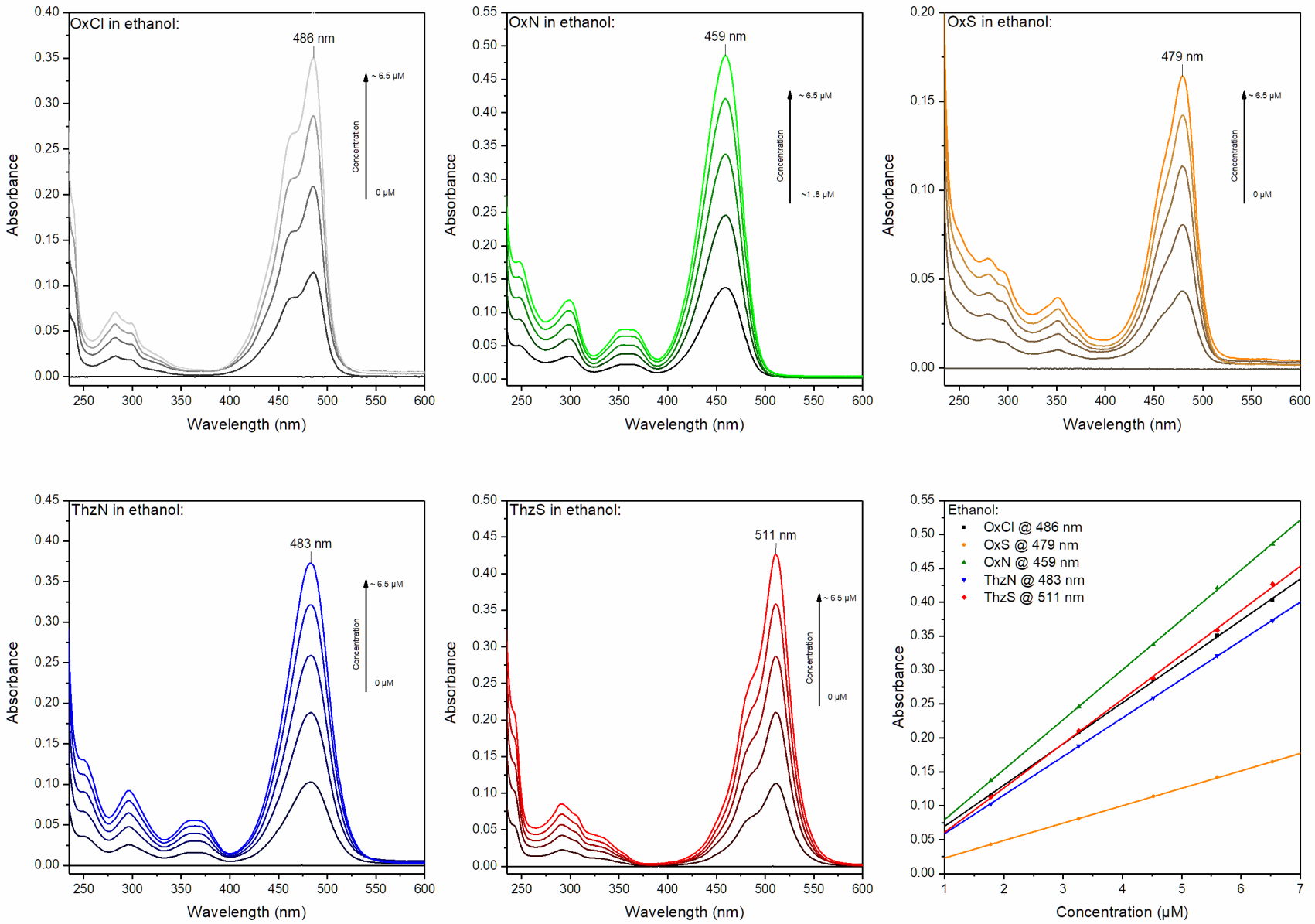


Fig. S15. UV-vis spectra of the synthesized dyes in five different concentrations in ethanol and linear fittings of the lowest energy maxima values.

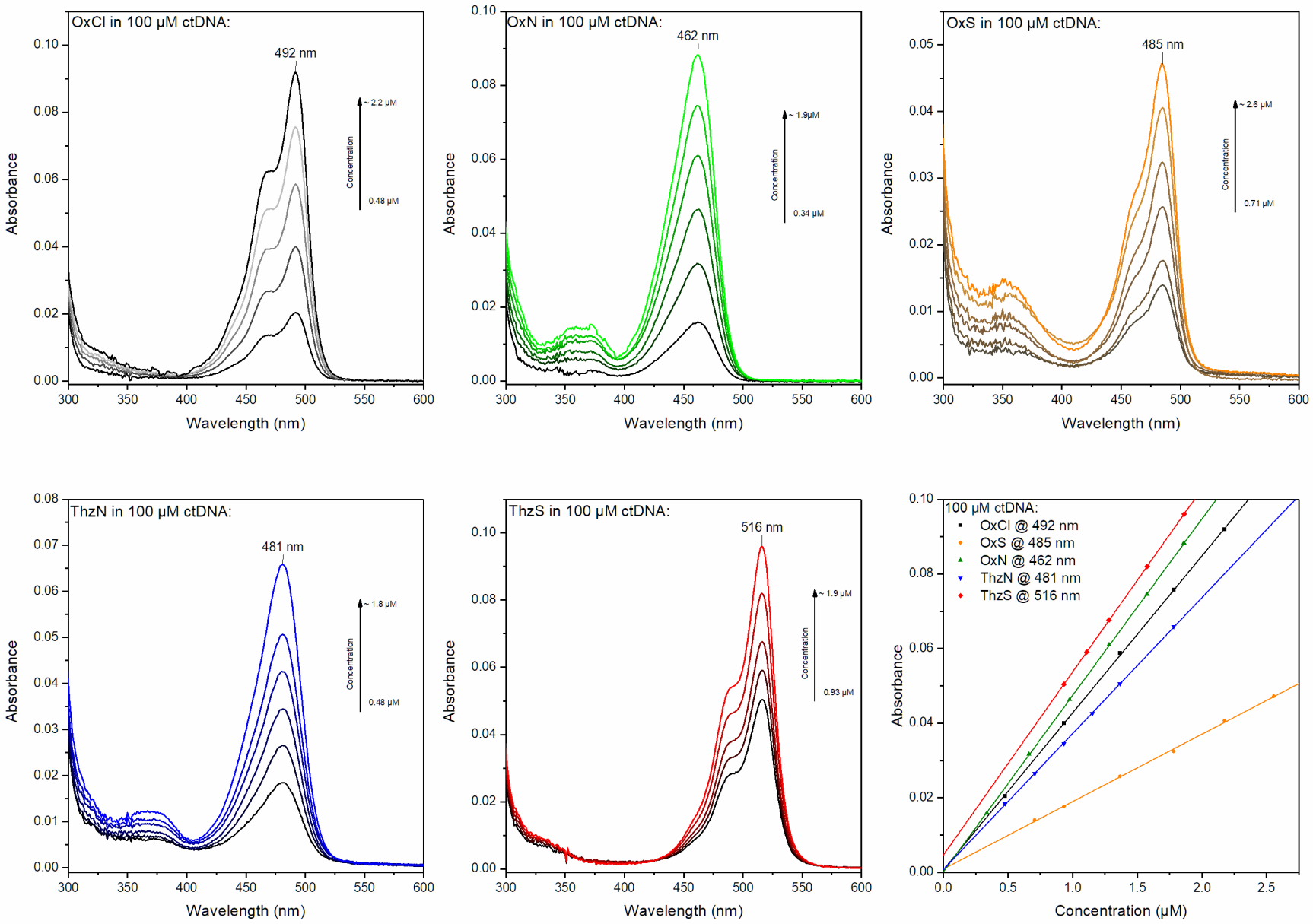


Fig. S16. UV-vis spectra of the synthesized dyes in five different concentrations in 100 µM ctDNA and linear fittings of the lowest energy maxima values.

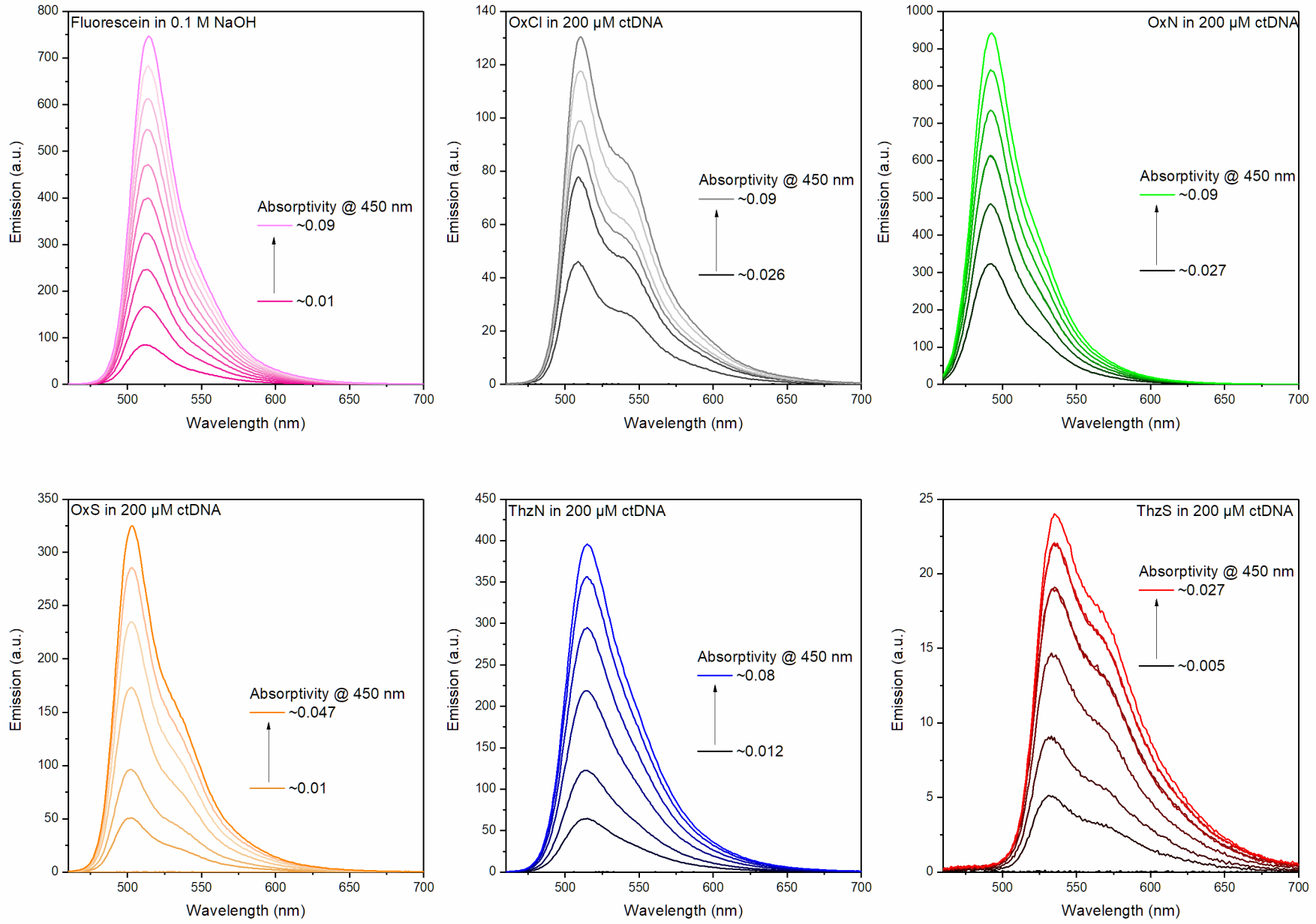


Fig. S17. Raw fluorescence spectra for fluorescein and synthesized dyes at different absorptivities. Integration of the spectra lead to the values used in Fig. S18.

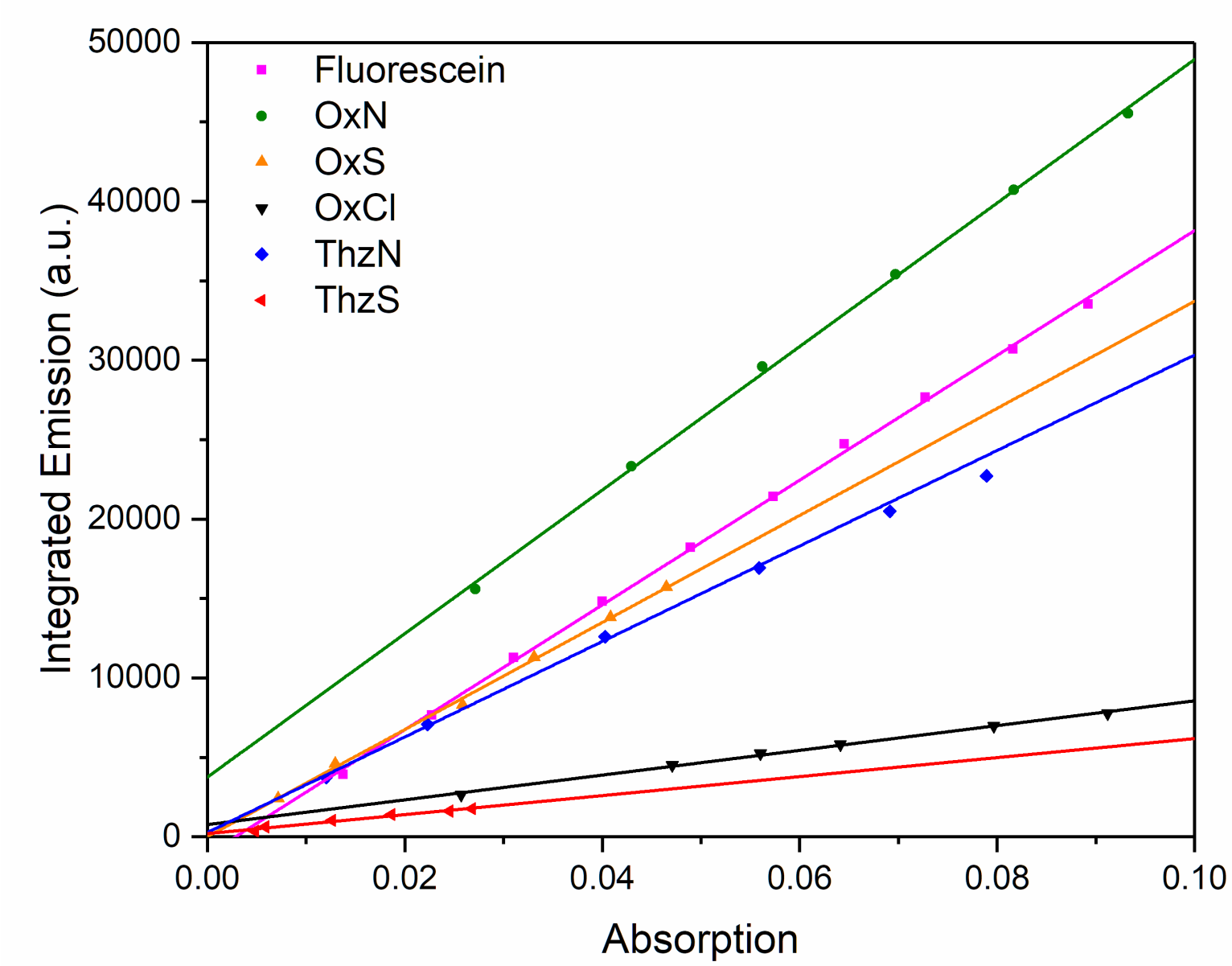


Fig. S18. Integrated emission values at different concentrations of respective dye *vs*. their absorption values at excitation wavelength 450 nm in high excess of ctDNA. Fluorescein was recorded in 0.1 M NaOH. Linear fitting gave the slope relative to the one given by fluorescein sample.

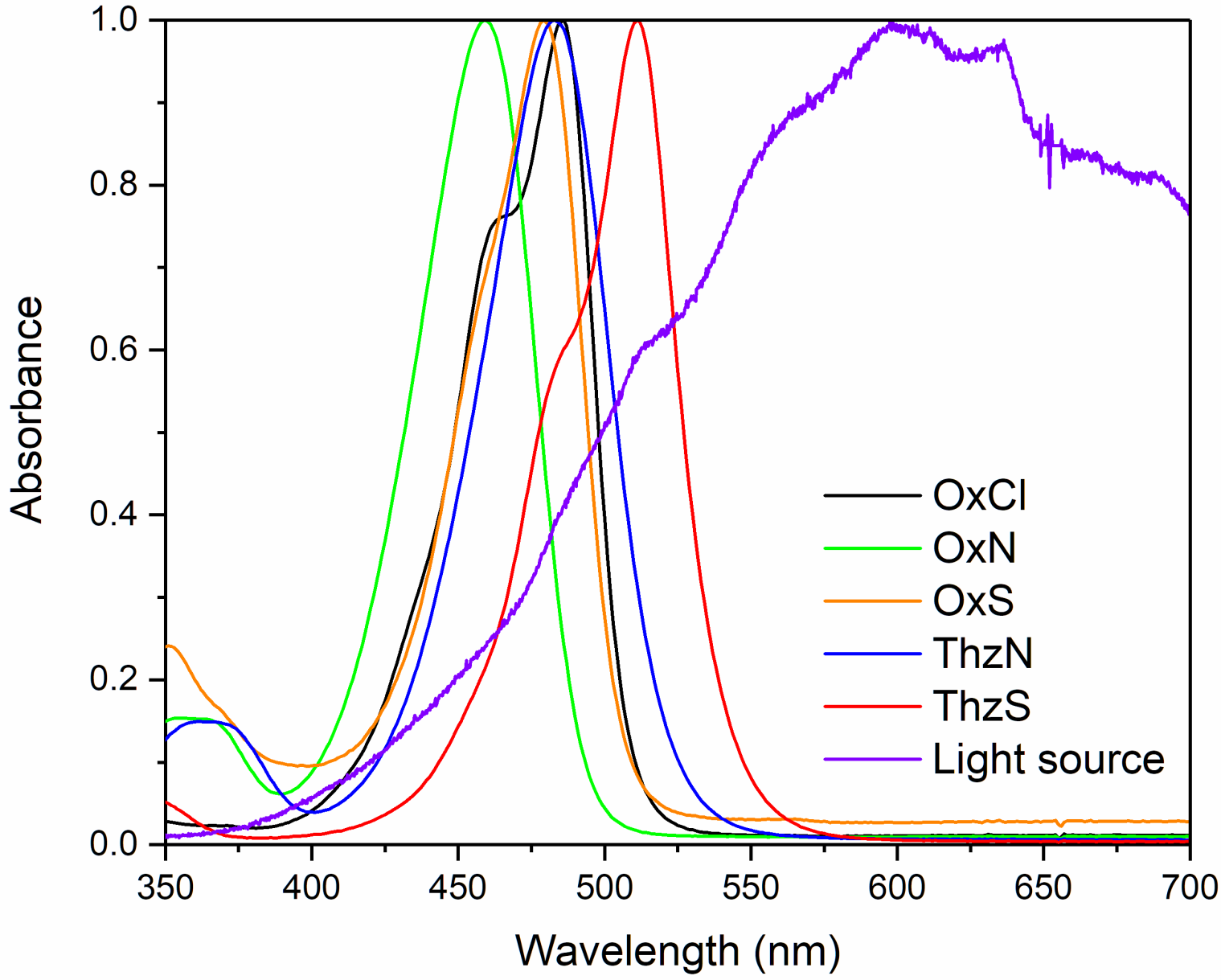


Fig. S19. Absorption spectra of the synthesized dyes overlaid with the 3300 K halogen lamp light source used for the photobleaching experiments.

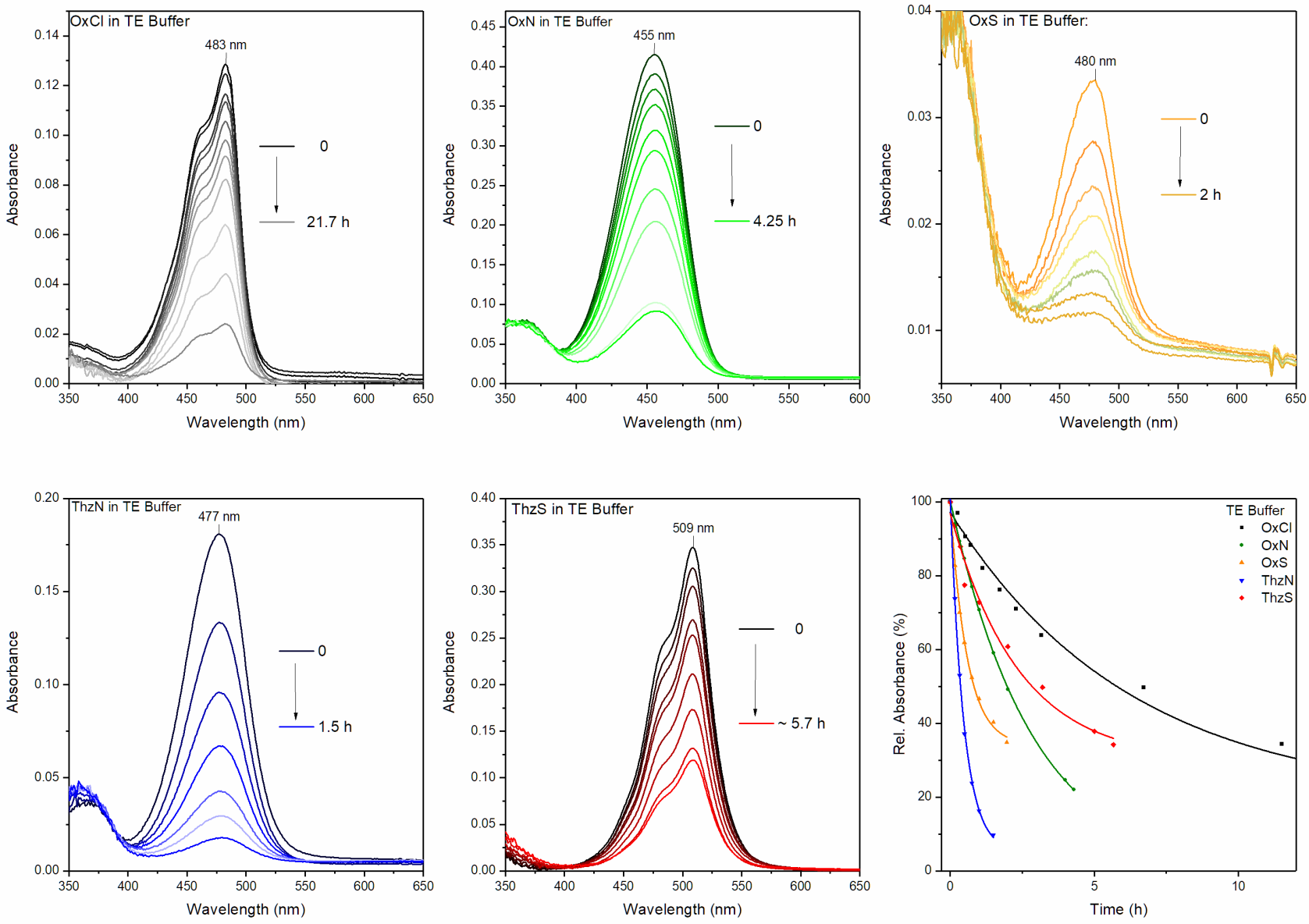


Fig. S20. Recorded absorption spectra of the synthesized dyes under a 22 mW/cm^2 halogen lamp exposure over time in TE buffer. Fitting exponential decay function to the lowest energy maxima values gave t½ values for each dye.

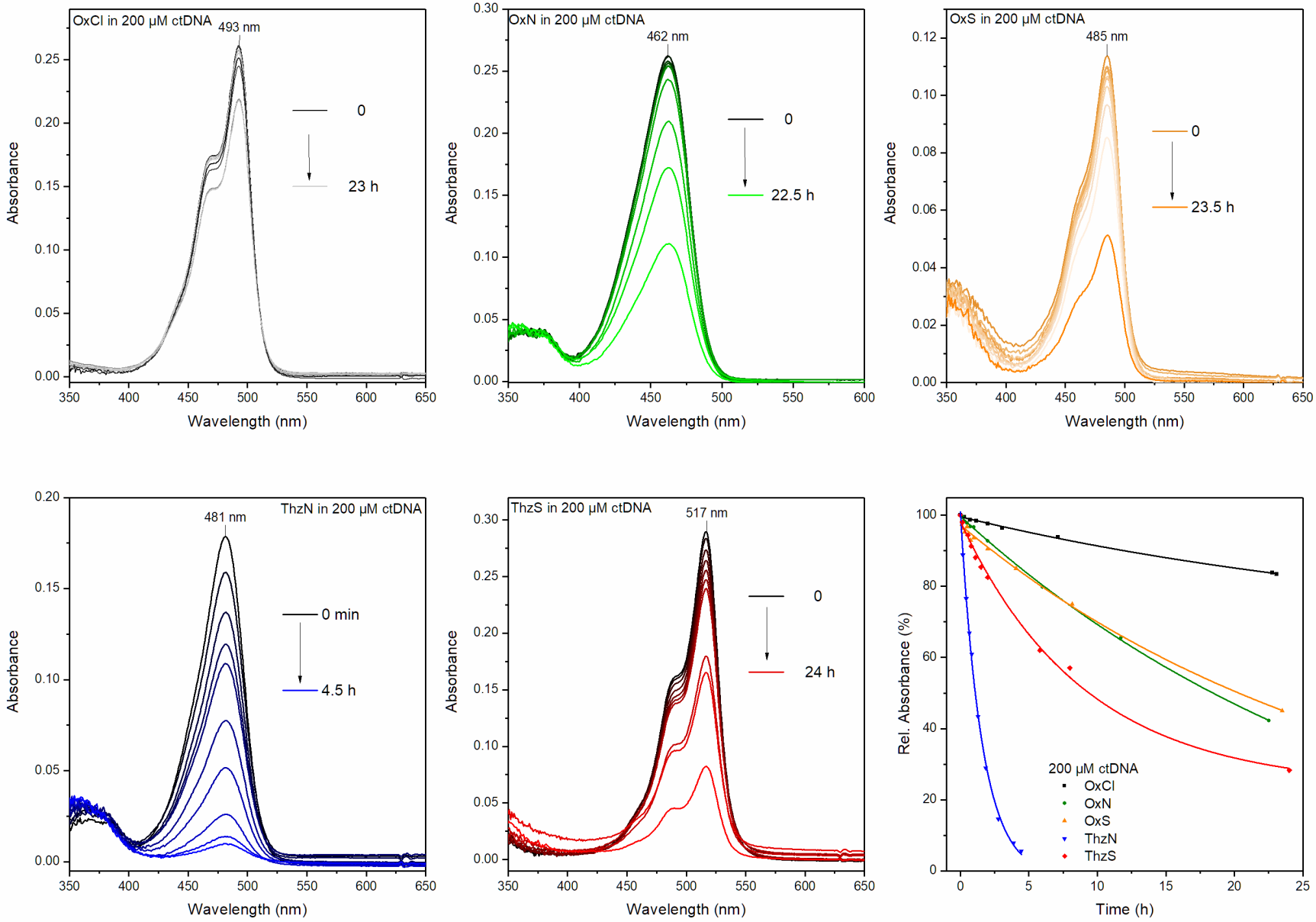


Fig. S21. Recorded absorption spectra of the synthesized dyes under a 22 mW/cm^2 halogen lamp exposure over time in 200 µM ctDNA. Fitting exponential decay function to the lowest energy maxima values gave $t_{½}$ values for each dye.

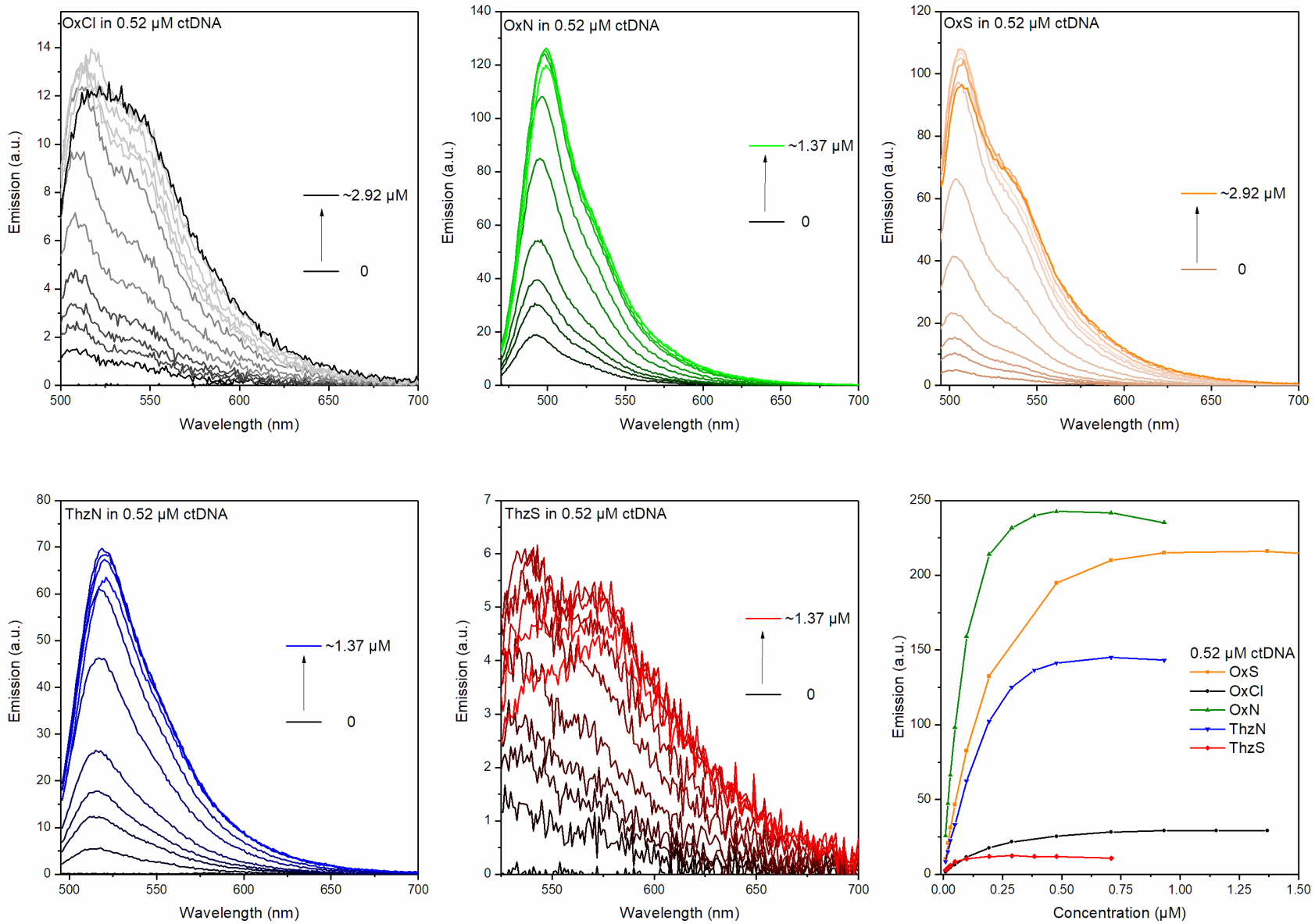


Fig. S22. Fluorescence spectra of the synthesized dyes with increasing concentration in 0.52 µM ctDNA solution and the corresponding emission maxima plotted as the function of concentration.

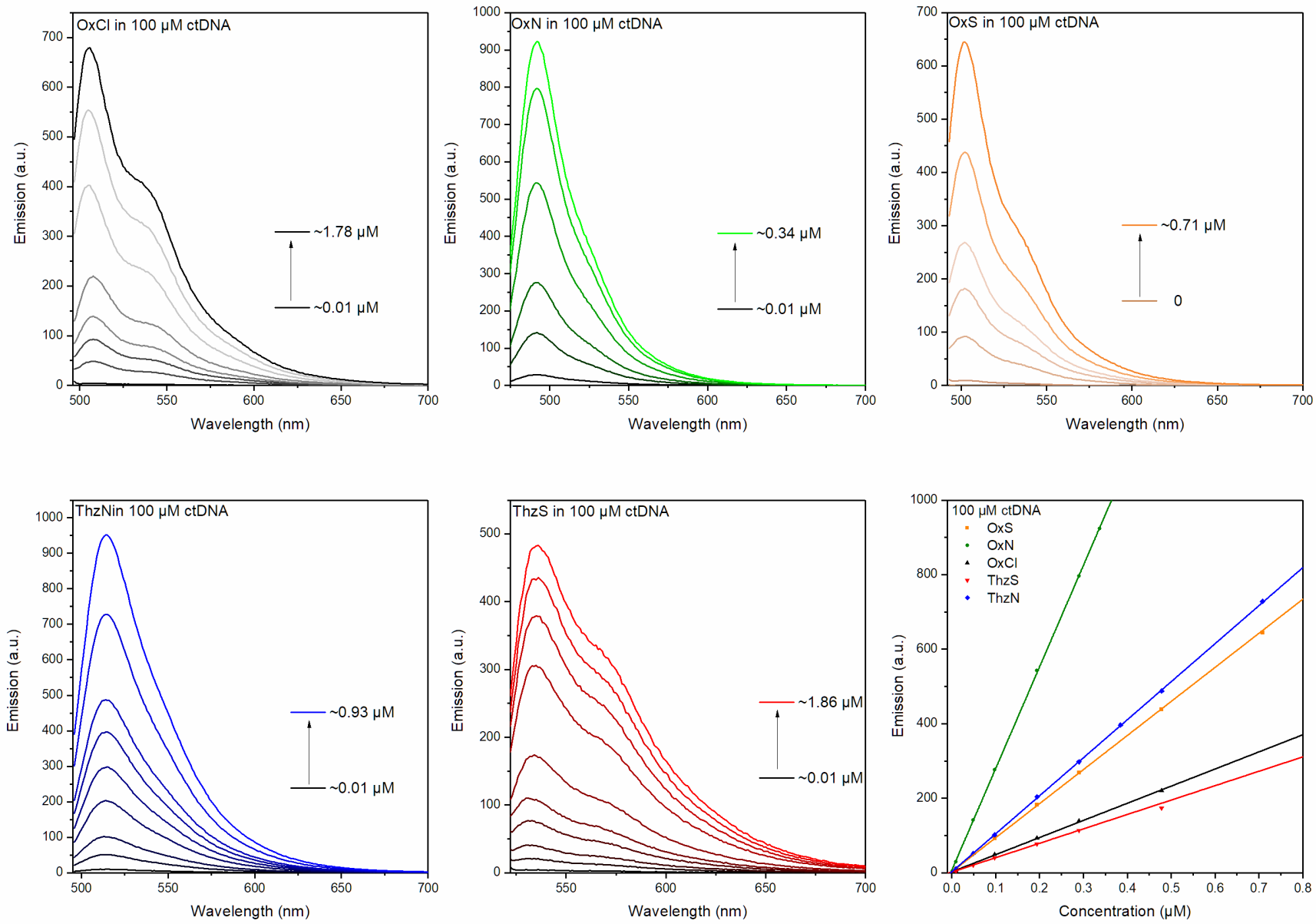


Fig. S23. Fluorescence spectra of the synthesized dyes with increasing concentration in 100 µM ctDNA solution and the corresponding emission maxima plotted as the function of concentration.

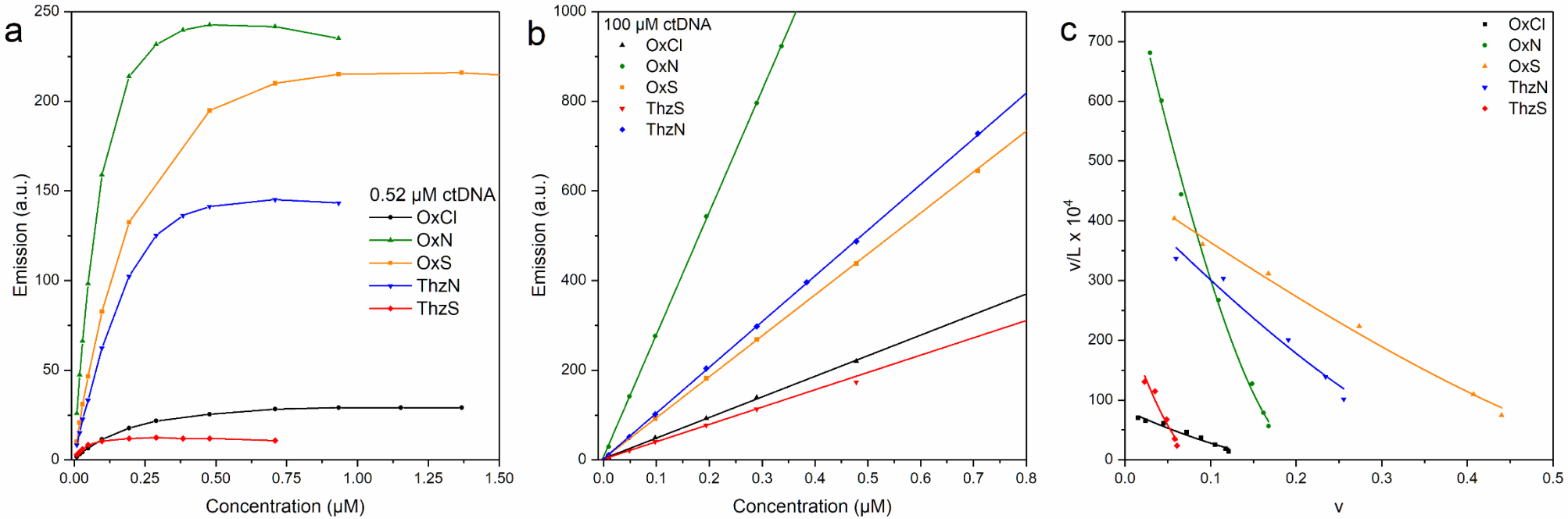


Fig. S24. Fluorescence emission maxima plotted with increasing dye concentration in a) 0.52 μM ctDNA (derived from Fig. S21) and b) 100 μM ctDNA (derived from Fig. S22). The processing of this data gave Scatchard plot c) depicting bound/free ratio as a function of bound dye molecules. The line corresponds to the fitting of the McGhee-von Hippel equation of non-cooperative DNA binding.

References

1 L. Ying, Nucleic acid detections and methods of their use, 2013/0137875, 2013, 1, 30–31.

2 M. Thompson, *Bioconjug. Chem.*, 2006, **17**, 507–513.

3 X. Li, S. R. Srinivasan, J. Connarn, A. Ahmad, Z. T. Young, A. M. Kabza, E. R. P. Zuiderweg, D. Sun and J. E. Gestwicki, *ACS Med. Chem. Lett.*, 2013, **4**, 1042–1047.

4 V. K. Saarnio, K. Salorinne, V. P. Ruokolainen, J. R. Nilsson, T.-R. Tero, S. Oikarinen, L. M. Wilhelmsson, T. M. Lahtinen and V. S. Marjomäki, *Dye. Pigment.*, 2020, **177**, 108282.

5 A. I. Dragan, R. Pavlovic, J. B. McGivney, J. R. Casas-Finet, E. S. Bishop, R. J. Strouse, M. A. Schenerman and C. D. Geddes, *J. Fluoresc.*, 2012, **22**, 1189–1199.

6 A. I. Dragan, J. R. Casas-Finet, E. S. Bishop, R. J. Strouse, M. A. Schenerman and C. D. Geddes, *Biophys. J.*, 2010, **99**, 3010–3019.

7 J. D. McGhee and P. H. von Hippel, *J. Mol. Biol.*, 1974, **86**, 469–489.