Supporting Information

Enhanced G-quadruplex DNA Stabilization and Telomerase Inhibition by Novel Fluorescein derived Salen and Salphen based Ni(II) and Pd(II) Complexes

Asfa Ali,[1] Mohini Kamra,[1] Soma Roy, [1] K. Muniyappa[3] and Santanu Bhattacharya[1,2]*

[1]Department of Organic Chemistry, Indian Institute of Science, Bangalore 560 012, India;

[2]Director's Research Unit, Indian Association for the Cultivation of Science, Kolkata 700 032, India;

[3]Department of Biochemistry, Indian Institute of Science, Bangalore 560 012, India.

**To whom correspondence should be addressed. E-mail: sb@orgchem.iisc.ernet.in*

Phone: (91)-80- 22932664; Fax: (91)-80- 22930529

Content **Page**

Comparison of the size of compounds vs. G-terads…….S3

Additional methods.....S4

Additional UV-Vis titrations..... S5-S6

Additional CD spectral titration data..... .....S7-S10

Additional fluorescence titration data.....S10-S11

Cell viability assay (short-term) data..... S11

Cell cycle (sub-G1 population) data..... S12

Additional cellular internalization images and graph.....S13-S14

DNA melting methods.....S14

Computational methods……..S14-S15

TRAP-Lig assay……..…...S15-S16

References…….....S17

^{1}H-NMR and ^{13}C-NMR spectra of the compounds.....S18-S23

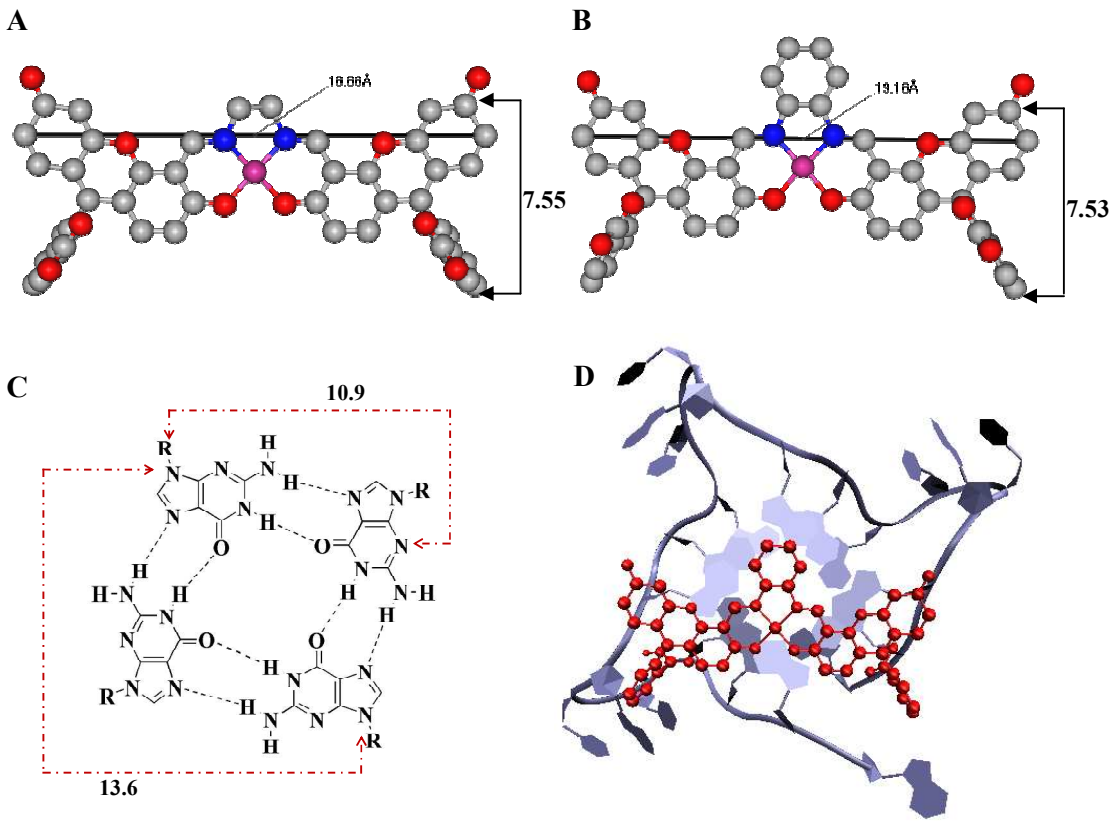

Figure S1. Optimized structures of (A) DFS-Ni and (B) DFSP-Ni at B3LYP/6-31G* level of theory with the indicated inter-atomic distances. (C) Structure of the G-tetrad with the dimensions as mentioned in ref. 1. (D) Stacking of DFSP-Ni on the plane of the G-tetrad determined by modeling studies.

Oligonucleotides. HPLC purified oligonucleotides (ODN) d[$G_3(T_2AG_3)_3$], abbreviated as Hum_{21}, were acquired from Sigma Genosys, Bangalore. High resolution sequencing gel confirmed their purity while the absorbance measurement at 260 nm resulted in the determination of the molar concentration of each ODN based on their molar extinction coefficient (ε_{260}) 215000.

G4 DNA formation. Single-stranded [5′-$G_3(T_2AG_3)_3$-3′] was incubated in 10 mM Tris-HCl, 0.1 M KCl or NaCl, pH 7.4 was first heated to 95 ^oC for 5 min followed by gradual cooling to room temperature over a period of 24 h. The formation of the G-quadruplex [Hum_{21}(KCl)/ Hum_{21}(NaCl)] DNA was validated by the characteristic signature in the circular dichroism spectroscopy and by PAGE.

UV-Vis titration of the ligands with preformed G-quadruplex DNA.

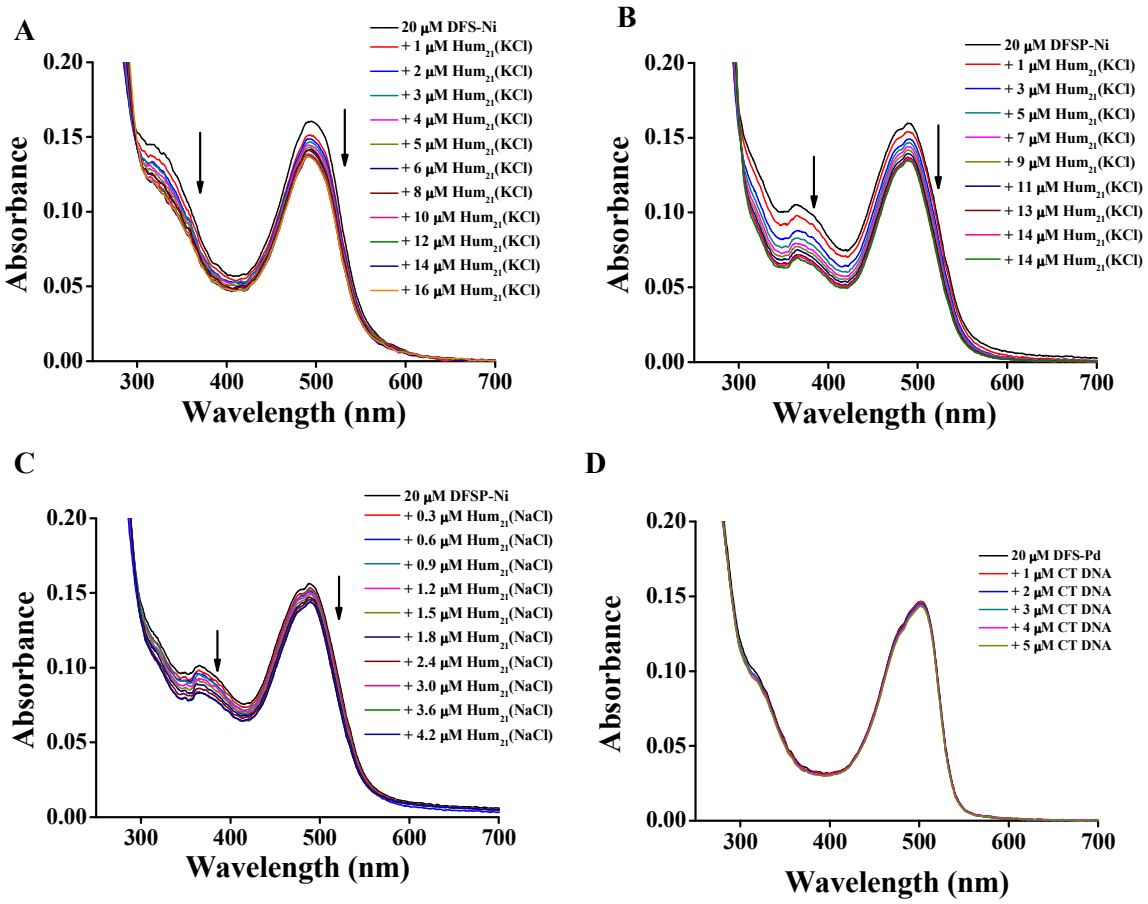

Figure S2. UV-Vis absorption spectral titrations of (A) **DFS-Ni** (B) **DFSP-Ni** with addition of Hum_{21}(KCl) G4 DNA and of (C) **DFSP-Ni** (D) **DFS-Pd** with addition of Hum_{21}(NaCl) G4 DNA and CT DNA, respectively. A buffer having 10 mM Tris-HCl, 100 mM KCl/NaCl, pH 7.4 have been used for titrations with Hum_{21} (KCl/NaCl) and a buffer having 10 mM Tris-HCl, 40 mM NaCl, pH 7.4 have been used for titrations with CT DNA.

All the titrations were performed in a Shimadzu model UV-2100 spectrophotometer. The extent of binding of the synthesized complexes to the pre-formed d[$G_3(T_2AG_3)_3$] quadruplex were determined by absorption spectral titration in 10 mM Tris-HCl buffer, pH 7.4 at 25 °C under physiological ionic condition (i.e. 100 mM KCl or 100 mM NaCl). Experiments with CT DNA and telomeric duplex DNA were carried out in 10 mM Tris-HCl, 40 mM NaCl, pH 7.4 at 25 °C. 20 μM of complex solution was titrated with gradual augmentation of DNA solution with incubation for 15 min before measurement. For each [DNA]/[complex], the fractional reduction in absorbance at 492 nm was normalized using equation 1.

$$\Delta A = (A_{free}-A)/(A_{free}-A_{sat}) \quad \text{.....1}$$

where A_{free} and A_{sat} are the absorbances of the free and fully bound complexes. The relative hypochromicity term ΔA directly reflects the fraction of bound complex α at each titration position.[2]

The binding ratio r, defined as $(C - C_f)$/[DNA], was determined where C denotes the total complex concentration and C_f is the concentration of free complex, calculated by using equation 2.

$$C_f = (1 - \alpha)C \quad \text{.....2}$$

The titration data were fitted into linear Scatchard equation 3.

$$r/C_f = K_a (n - r) \quad \text{.....3}$$

where n is the site exclusion parameter signifying the number of complex molecules bound per G-quadruplex DNA. The intrinsic equilibrium binding constant Ka was determined from the plot of r/C_f versus r using *OriginPro 8*.

Circular Dichroism Spectroscopy.

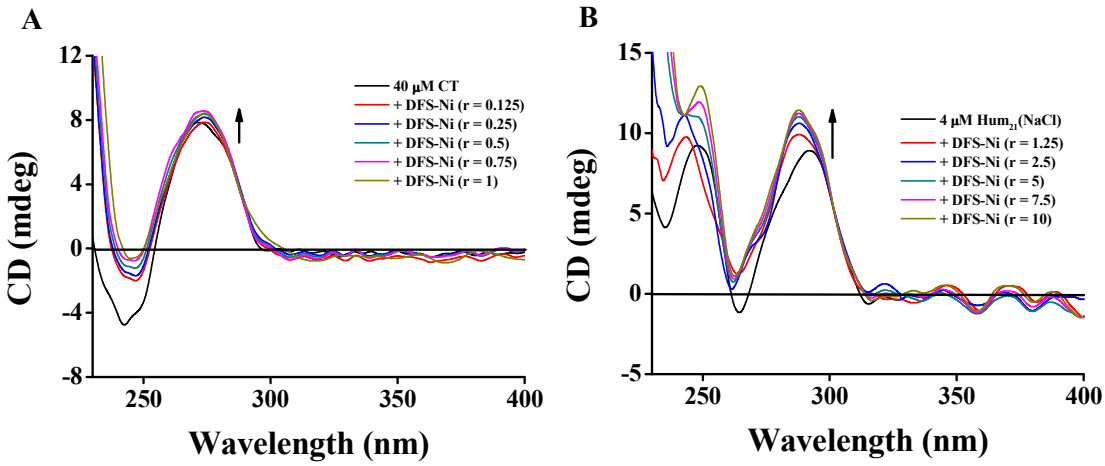

Figure S3. CD spectral titrations of (A) 40 µM CT DNA and (B) 4 µM Hum_{21}(NaCl) G4 DNA with increasing concentration of **DFS-Ni** in a buffer having 10 mM Tris-HCl, 40 mM NaCl/100 mM NaCl, pH 7.4, respectively.

The CD spectra were recorded on a JASCO J-810 CD spectropolarimeter with a Peltier temperature controller at a scanning speed of 50 nm/min with a quartz cell of 10 mm path-length. To a 4 µM pre-formed Na^+ or K^+ induced G-quadruplex DNA Hum_{21}(KCl) or Hum_{21}(NaCl), respectively, aliquots of the complex solution prepared in the respective buffer solutions were added with an equilibration time of 15 min and the experiment was pursued until a saturation was obtained.

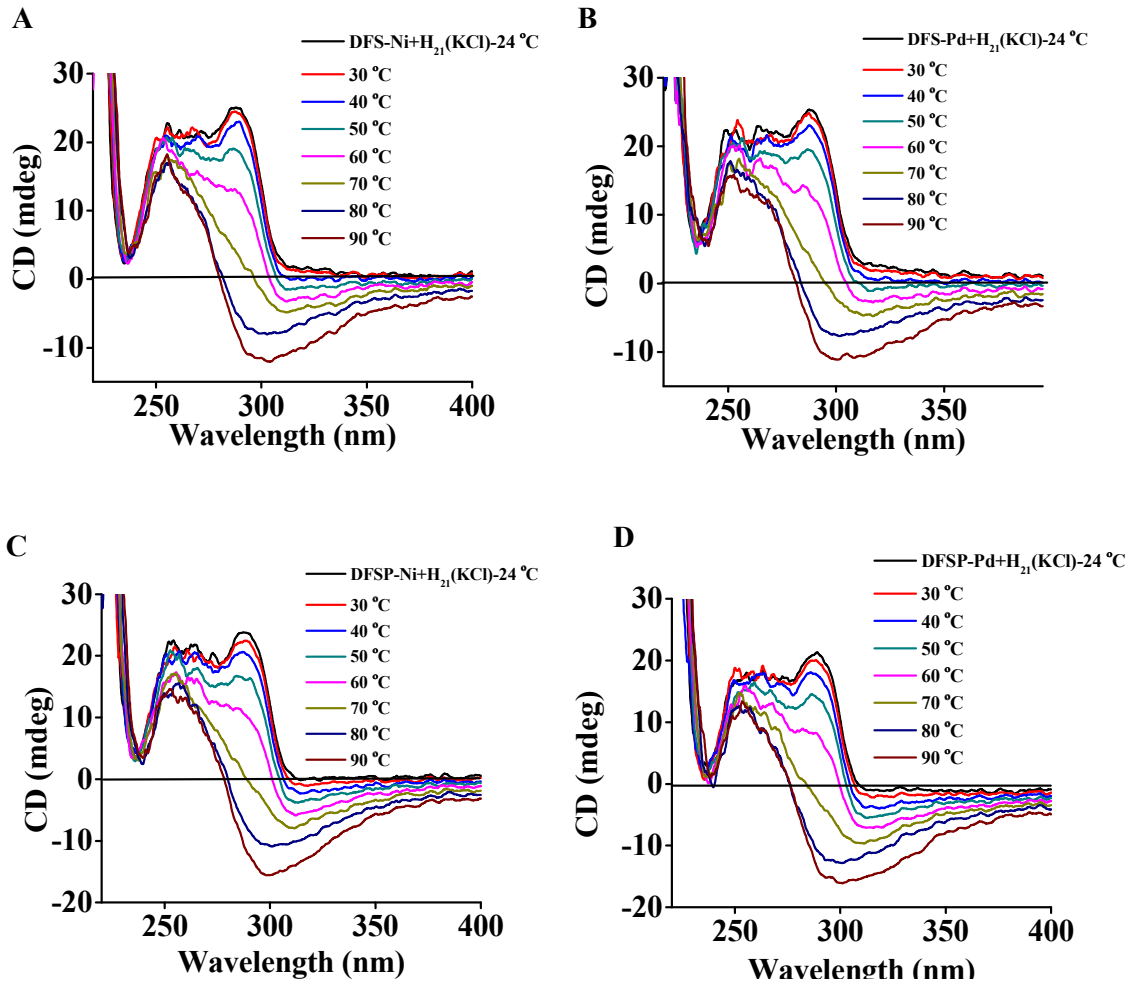

Figure S4. Variable temperature CD spectral titrations of (A) **DFS-Ni** (B) **DFS-Pd** (C) **DFSP-Ni**, and (D) **DFSP-Pd** with 4 μM Hum_{21}(KCl) G4 DNA (r = 2, where r = [compound]/[DNA]) in a buffer having 10 mM Tris-HCl, 100 mM KCl, pH 7.4.

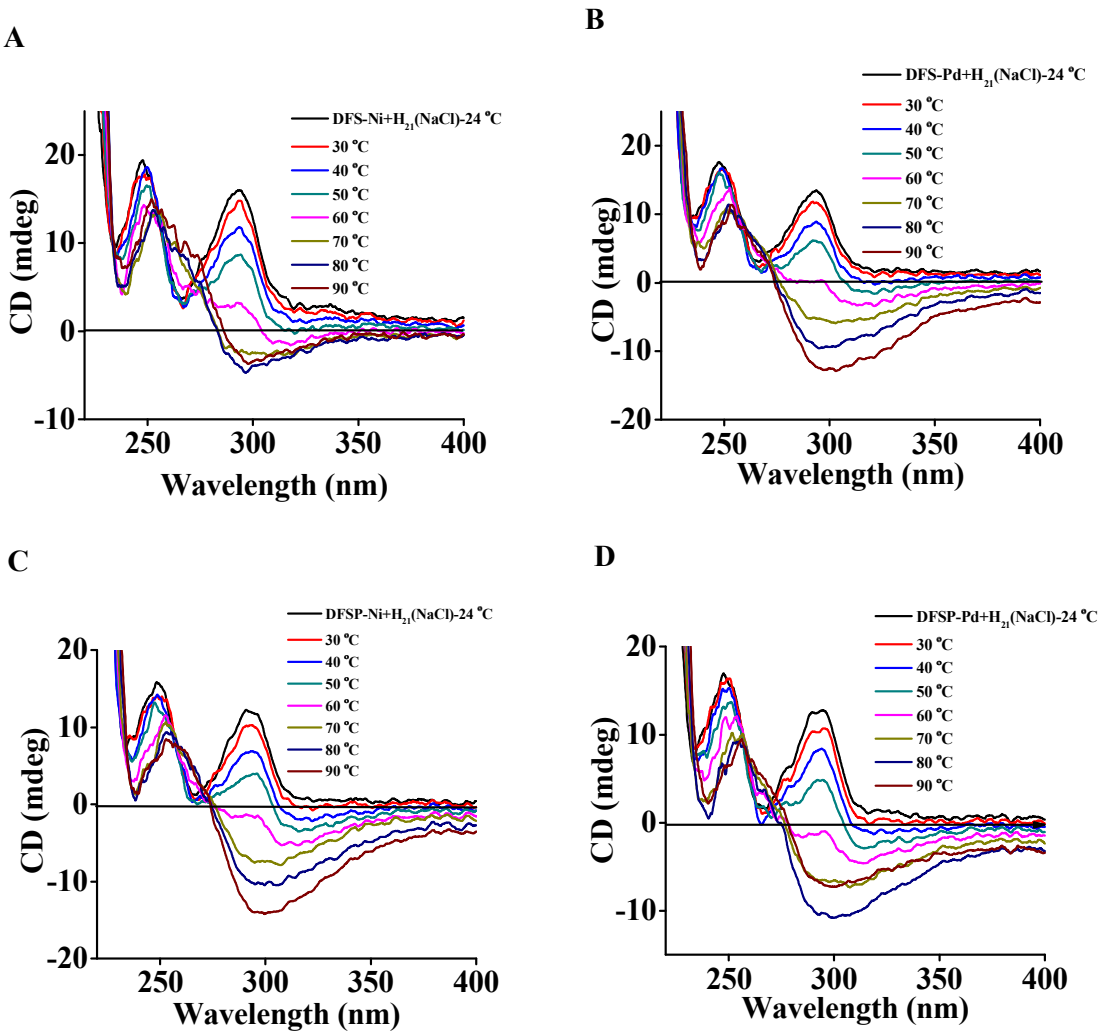

Figure S5. Variable temperature CD spectral titrations of (A) **DFS-Ni** (B) **DFS-Pd** (C) **DFSP-Ni**, and (D) **DFSP-Pd** with 4 μM Hum_{21}(NaCl) G4 DNA (r = 2, where r = [compound]/[DNA]) in a buffer having 10 mM Tris-HCl, 100 mM KCl, pH 7.4.

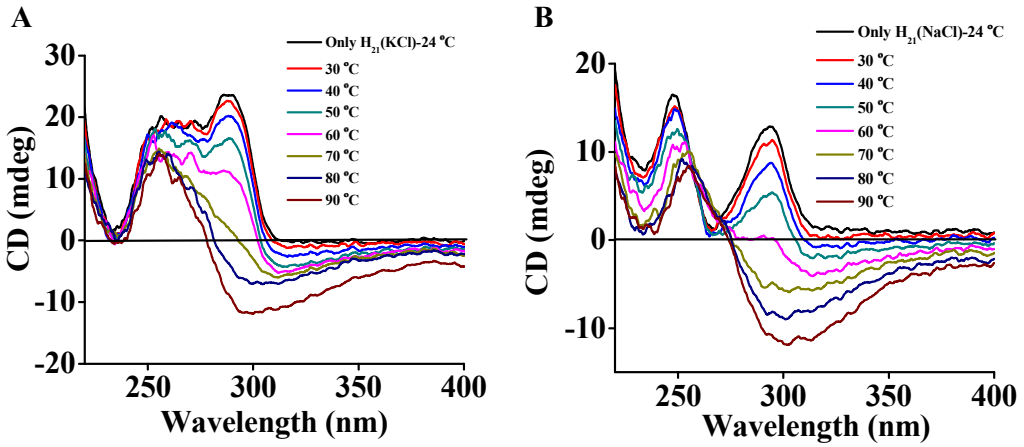

Figure S6. Variable temperature CD spectral titrations of only 4 μM (A) Hum_{21}(KCl) and (B) Hum_{21}(NaCl) G4 DNA (in a buffer having 10 mM Tris-HCl, 100 mM KCl/NaCl, pH 7.4.

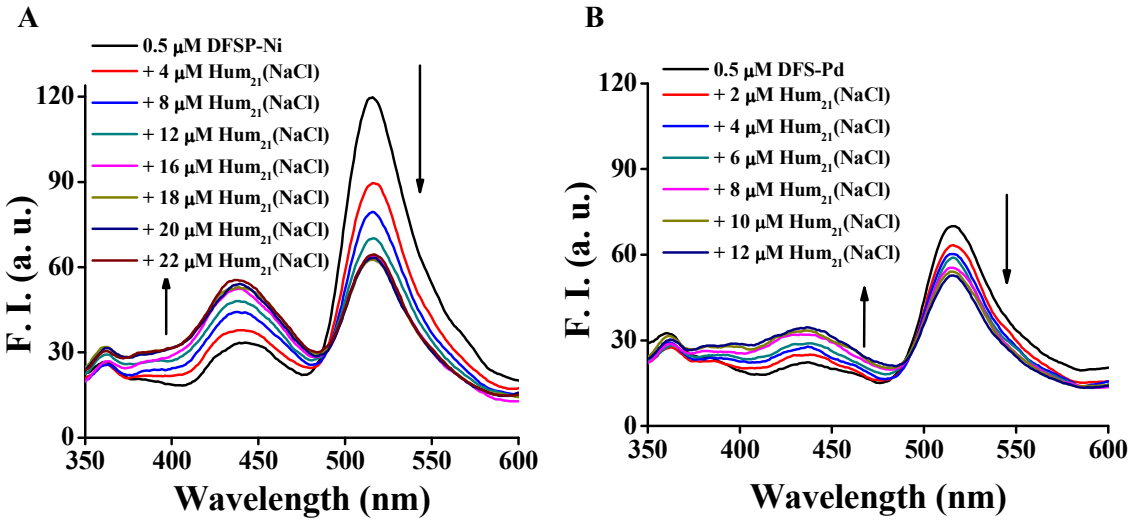

Figure S7. Fluorescence titrations of (A) 0.5 μM **DFSP-Ni** with Hum_{21}(NaCl) and (B) 0.5 μM **DFS-Pd** with Hum_{21}(NaCl) G4 DNA with saturation at [G4 DNA]/[ligand] ratio of 40 and 24, respectively in a buffer having 10 mM Tris-HCl, 100 mM KCl/NaCl, pH 7.4.

Table S1. Stern-Volmer quenching constants (K_a) of the metal complexes with the pre-formed G-quadruplex DNA and CT DNA as determined by fluorescence spectroscopy.

Metal Complex	K_a (10^4 M^{-1})		
	Hum_{21}(KCl)[a]	Hum_{21}(NaCl)[a]	*ds*-DNA[b]
DFS-Ni	0.2 ± 0.01	4.9 ± 0.7	3.9 ± 0.3
DFS-Pd	2.5 ± 0.01	2.6 ± 0.2	2.3 ± 0.2
DFSP-Ni	7.4 ± 0.2	4.9 ± 0.3	4.1 ± 0.2
DFSP-Pd	4.2 ± 0.2	4.6 ± 0.4	3.0 ± 0.3

[a]Fluorescence spectral titrations were performed with the Hum_{21}(KCl) and Hum_{21}(NaCl) G4 DNA in a buffer having 10 mM Tris-HCl, 100 mM KCl/NaCl, pH 7.4. Fluorescence spectral titrations were performed with CT DNA in a buffer having 10 mM Tris-HCl, 40 mM NaCl, pH 7.4.

Table S2. Effect of metal complexes on cell viability after a short-term (72 h) exposure[a]

Metal Complex	HEK 293T	A549	HeLa	HEK 293	HFF
DFS-Ni	25 ± 5	37 ± 4	>100	>100	>100
DFS-Pd	>100	21 ± 2	>100	>100	40 ± 2
DFSP-Ni	14 ± 5	>100	>100	>100	70 ± 5
DFSP-Pd	75 ± 5	>100	>100	>100	>100

[a]Cells were treated with varying concentrations of complexes in triplicates in individual experiments and the results are based on at least three independent experiments. The values are in μM.

Table S3. Determination of sub-G1 (apoptotic) cell population on metal complex treatment[a]

Sample	% Apoptotic Cells	
	HEK 293T	**A549**
Cells alone	15 ± 2	19 ± 1
DFS-Ni	42 ± 1	24 ± 1
DFS-Pd	35 ± 2	29 ± 2
DFSP-Ni	33 ± 2	23 ± 2
DFSP-Pd	42 ± 1	28 ± 3

[a]Human embryonic kidney transformed cells (HEK 293T) cells treated with ligands for 48 h and determined by WinMDI public domain software.

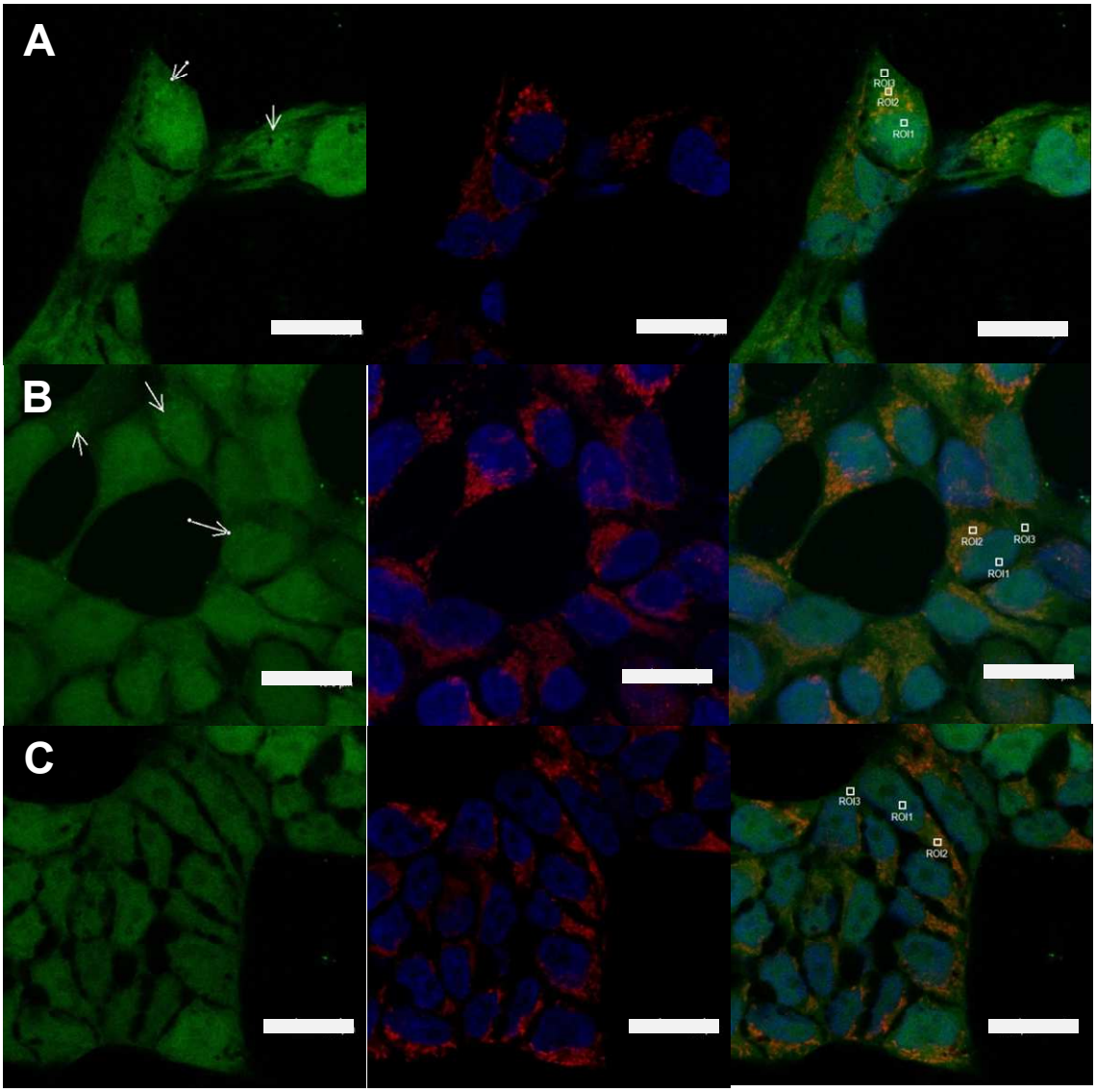

Figure S8. (A-C) Representative confocal microscopic images (3 regions) of HEK 293T cells on treatment with DFSP-Ni for 24 h at near IC50 concentration. DAPI and MitoTracker® Red CMXRos were used as the nuclear and mitochondrial counterstains, respectively. Panels (left to right) represent, metal complex fluorescence (green), overlay of green with DAPI nuclear (blue) and MitoTracker® Red CMXRos mitochondrial (red), respectively. Scale bar = 20μm.

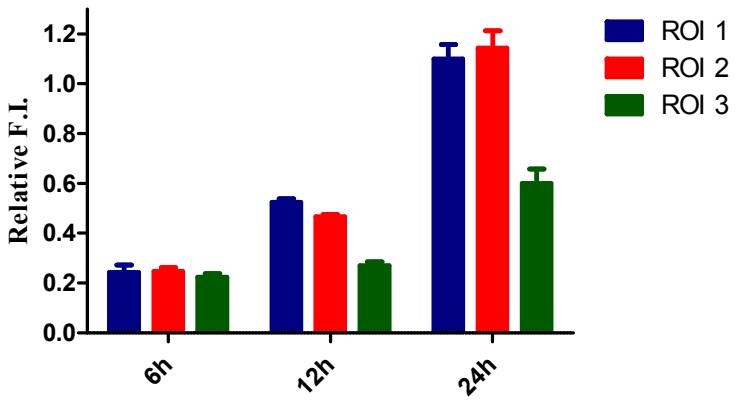

Figure S9. Plot of relative fluorescence intensity vs. time obtained from the images taken at different time points. Selection of region of interest (ROI) in nucleus (ROI 1), mitochondria (ROI 2), cytoplasm (ROI 3).

DNA Melting Experiment. The CD melting experiments were performed on a JASCO J-810 CD spectropolarimeter equipped with a Peltier temperature controller with a quartz cell of 10 mm path-length. A temperature range of 25 to 90 °C and monitored at an interval of 0.5 °C/min. All the experiments were repeated twice and the error ± 0.5 °C has been reported. Origin 8.0 software was used for plotting the data. Normalization of the CD intensity was done to the range of 0 and 1. A 4 μM Hum_{21}(KCl) G4 DNA and complex solutions (8 μM) were used after dilution in a buffer having 10 mM Tris-HCl, 100 mM KCl, pH 7.4.

Computational studies. Gaussian 03 suite program was used to perform molecular energy optimizations with B3LYP/6-31G* level of theory. The initial PDB structures of the

complexes were thereafter obtained from optimized structure by using Open Babel software. The crystal structure of the parallel propeller G-quadruplex DNA (PDB 1KF1) was used as the macromolecules in the docking studies with the complexes using *Autodock 4.0* software.[3] Docking studies were performed using a grid box dimension (126 × 126 × 126 points, spacing 0.375 Å) by keeping the center at the macromolecule. The calculations utilized the Lamarkian genetic algorithm (LGA) program with a random initial population of size 150, maximum number of energy evaluations of 2500000, maximum number of generations 27000, and a mutation rate of 0.02. Hundred independent docking runs were performed for the respective complex and a root-mean-square cut-off of 0.5 Å was set for the study. The energetically minimum structure with maximum clustering pattern was considered as the most stable structure of the G4 DNA-complex.

TRAP-LIG Assay. We performed a modified TRAP-LIG assay as reported.[4,5] It broadly involves three steps.

Step 1. A master mix containing 0.1 μg of TS forward primer (5'-AAT CCG TCG AGC AGA GTT-3'), TRAP buffer (20 mM Tris-HCl, pH 8.3, 68 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, and 0.05% [v/v] Tween 20), dNTPs (125 μM each), and protein extract (500 ng/sample)was diluted in CHAPS lysis buffer (10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.5% CHAPS, 10% glycerol, 5 mM β-mercaptoethanol, and 0.1 mM 4-(2-aminoethyl)-benzene sulfonyl fluoride to perform the initial elongation step. This master mix was then added to negative control containing no complex and freshly prepared complex solutions at different concentrations. The initial elongation step was then performed at 37 °C for 30 min followed by heating at 94 °C for 5 min to deactivate the telomerase and the mixture is then maintained at 20 °C.

Step 2. The elongated product was purified along with removal of the bound and free complexes by using the QIA kit (Qiagen) according to the manufacturer's instructions. It purifies both the double- and single-stranded ODNs from 17 bases in length and has a high-salt buffer that binds the negatively charged ODNs to the positively charged spin tube membrane. This occurs through centrifugation which ensures that all other components along with the positively charged and neutral complex molecules are eluted. Washing was performed with PCR-grade water to remove any impurity before the elution of DNA using a low-salt concentration solution. The samples were freeze-dried after purification and then re-dissolved in PCR-grade water prior to second amplification step at room temperature.

Step 3. The final step involves the PCR amplification of the purified samples. Here, a second PCR master mix was prepared which comprised 1 μM ACX reverse primer (5'-GCG CGC $[CTTACC]_3$ CTA ACC -3'), 0.1 μg TS forward primer (5'-AAT CCG TCG AGC AGA GTT-3'), TRAP buffer, 5 μg BSA, 0.5 mM DNTPs, and 2 U *Taq* polymerase. A 6 μL of the master mix was then added to the purified and extended samples followed by amplification for 30 cycles of 30 s at 94 °C and 30 s at 59 °C. After the completion of the reaction, the products were loaded onto a 10% polyacrylamide gel (19:1) in TBE (0.5 X) against 1800 V. The gels were then transferred to a Whatman (3 mm) paper, dried under vacuum (80 °C), and read using a phosphoimager 840 (Amersham). All the measurements were made in triplicates with respect to a negative control run using equivalent TRAP-PCR conditions but without the protein extract to ensure that the ladders observed did not result from artefacts of the PCR reaction.

References.

1. Parkinson, G. N., Lee, M. P., and Neidle, S. (2002) Crystal structure of parallel quadruplexes from human telomeric DNA. *Nature 417*, 876-880.
2. Peacocke, A. R., and Skerrett, J. N. H. (1956) The interaction of aminoacridines with nucleic acids. *Trans. Faraday Soc. 52*, 261-279.
3. Morris, G. M., Goodsell, D. S., Halliday, R. S., Huey, R., Hart, W. E., Belew, R. K., and Olson, A. J. (1998) Automated docking using a Lamarckian genetic algorithm and an empirical binding free energy function. *J. Comput. Chem. 19*, 1639-1662.
4. De Cian, A., Cristofari, G., Reichenbach, P., De Lemos, E., Monchaud, D., Teulade-Fichou, M. P., Shin-ya, K., Lacroix, L., Lingner, J., and Mergny, J. L. (2007) Reevaluation of telomerase inhibition by quadruplex ligands and their mechanisms of action. *Proc. Natl. Acad. Sci. U.S.A. 104*, 17347-17352.
5. Reed, J., Gunaratnam, M., Beltran, M., Reszka, A. P., Vilar, R., and Neidle, S. (2008) TRAP-LIG, a modified telomere repeat amplification protocol assay to quantitate telomerase inhibition by small molecules. *Anal. Biochem. 380*, 99-105.

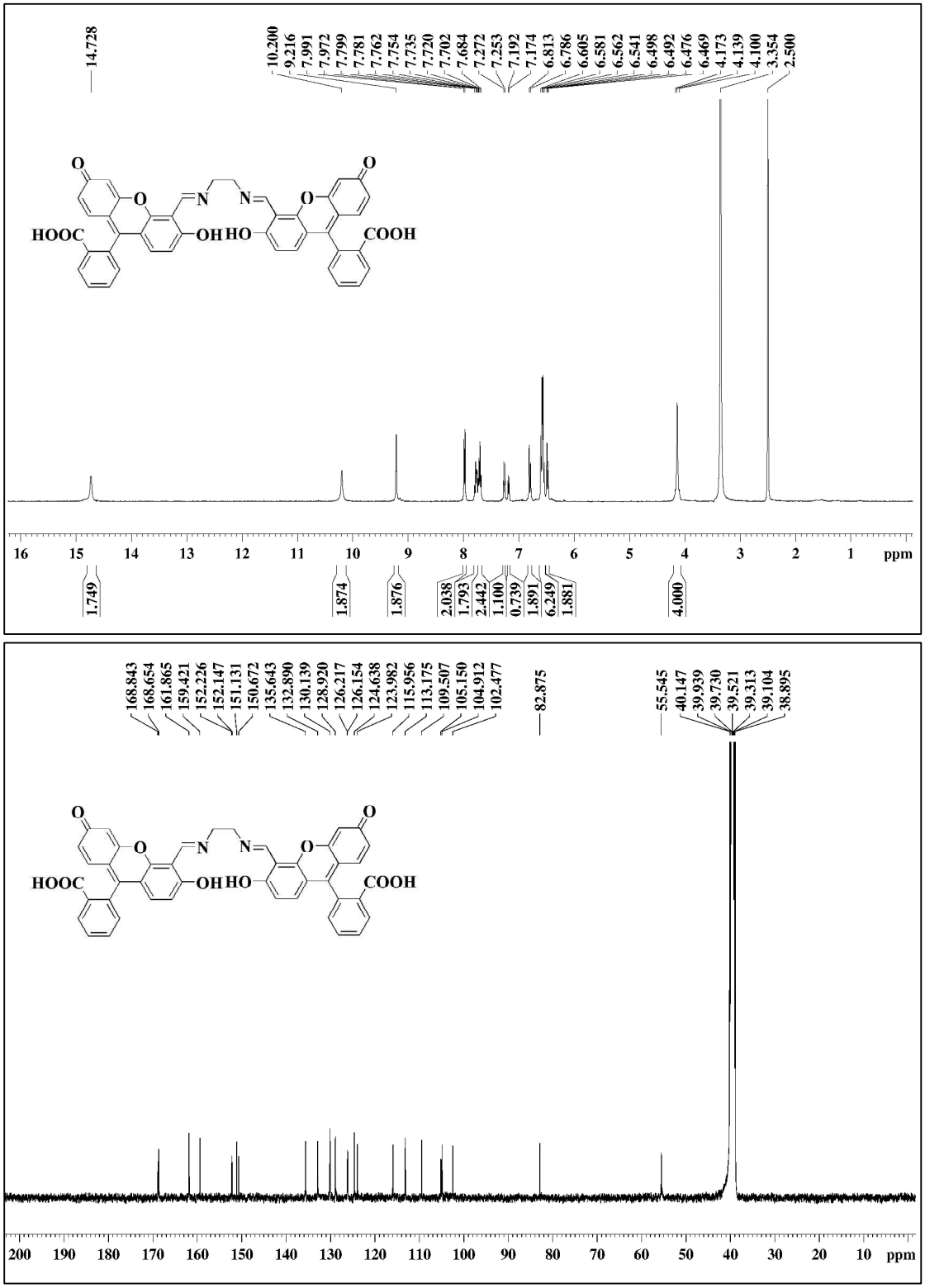

Figure S10. (A) ^{1}H-NMR and (B) ^{13}C-NMR of compound **6**.

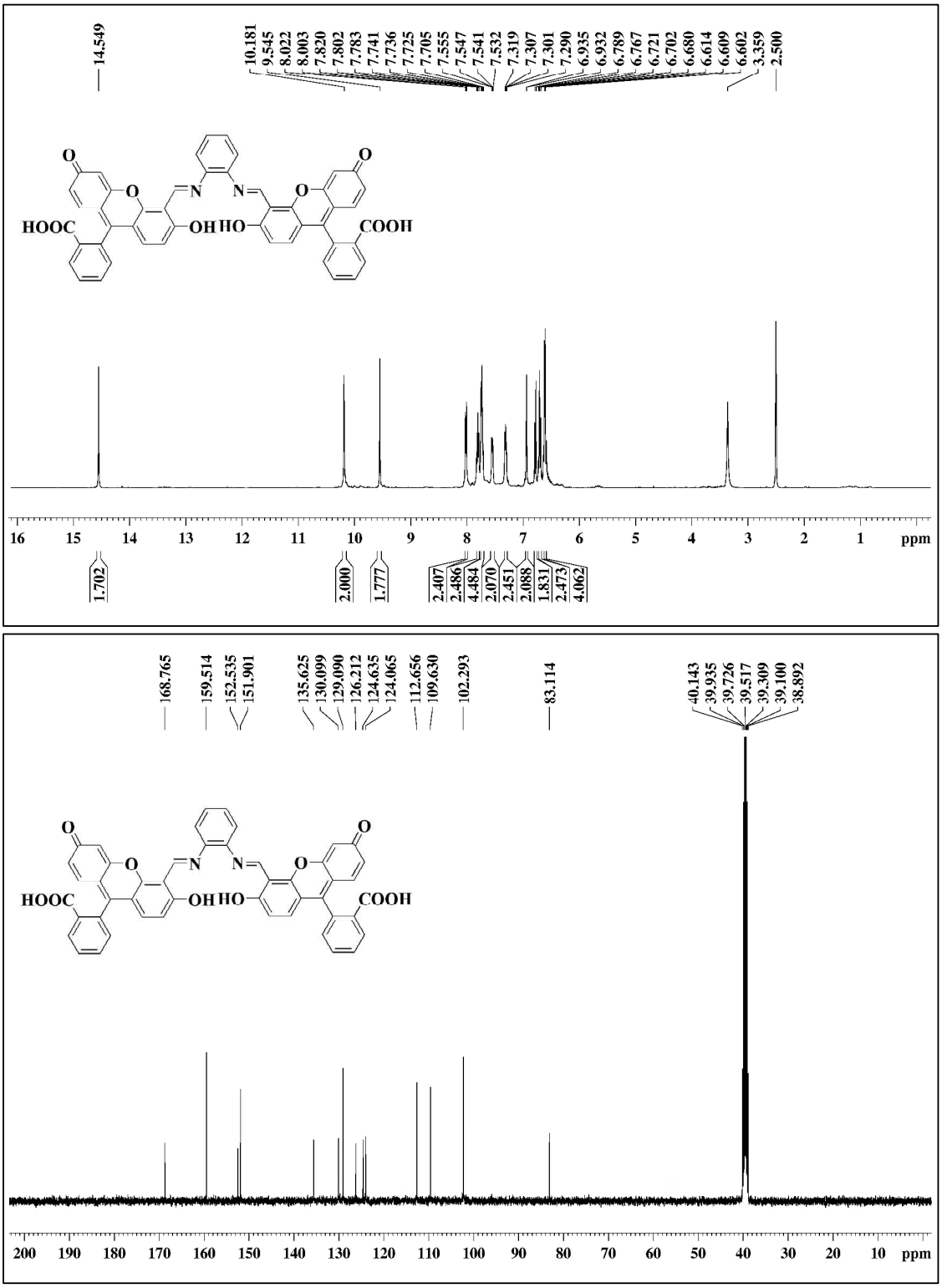

Figure S11. (A) ^{1}H-NMR and (B) ^{13}C-NMR of compound **7**.

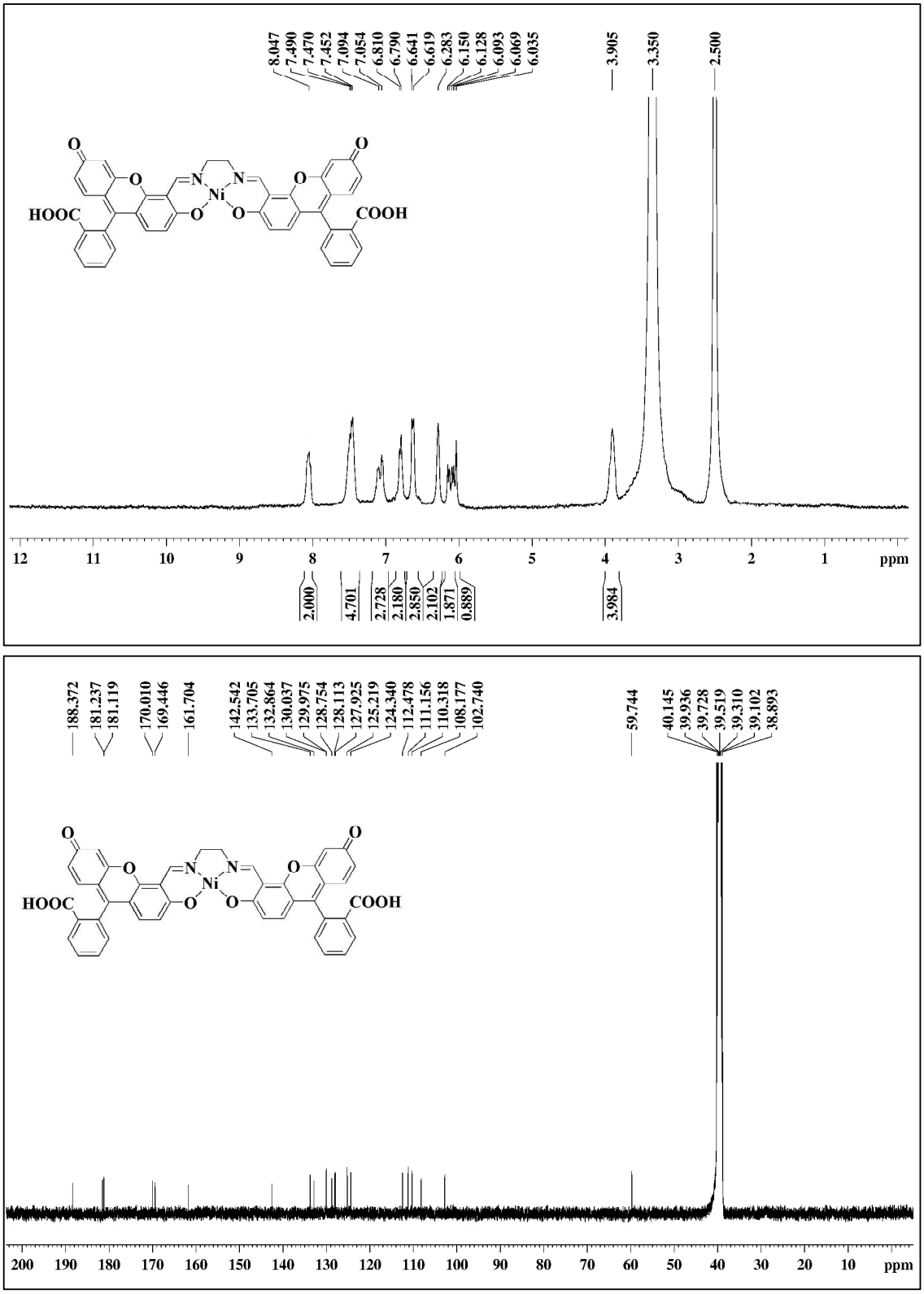

Figure S12. (A) ^{1}H-NMR and (B) ^{13}C-NMR of compound **1**.

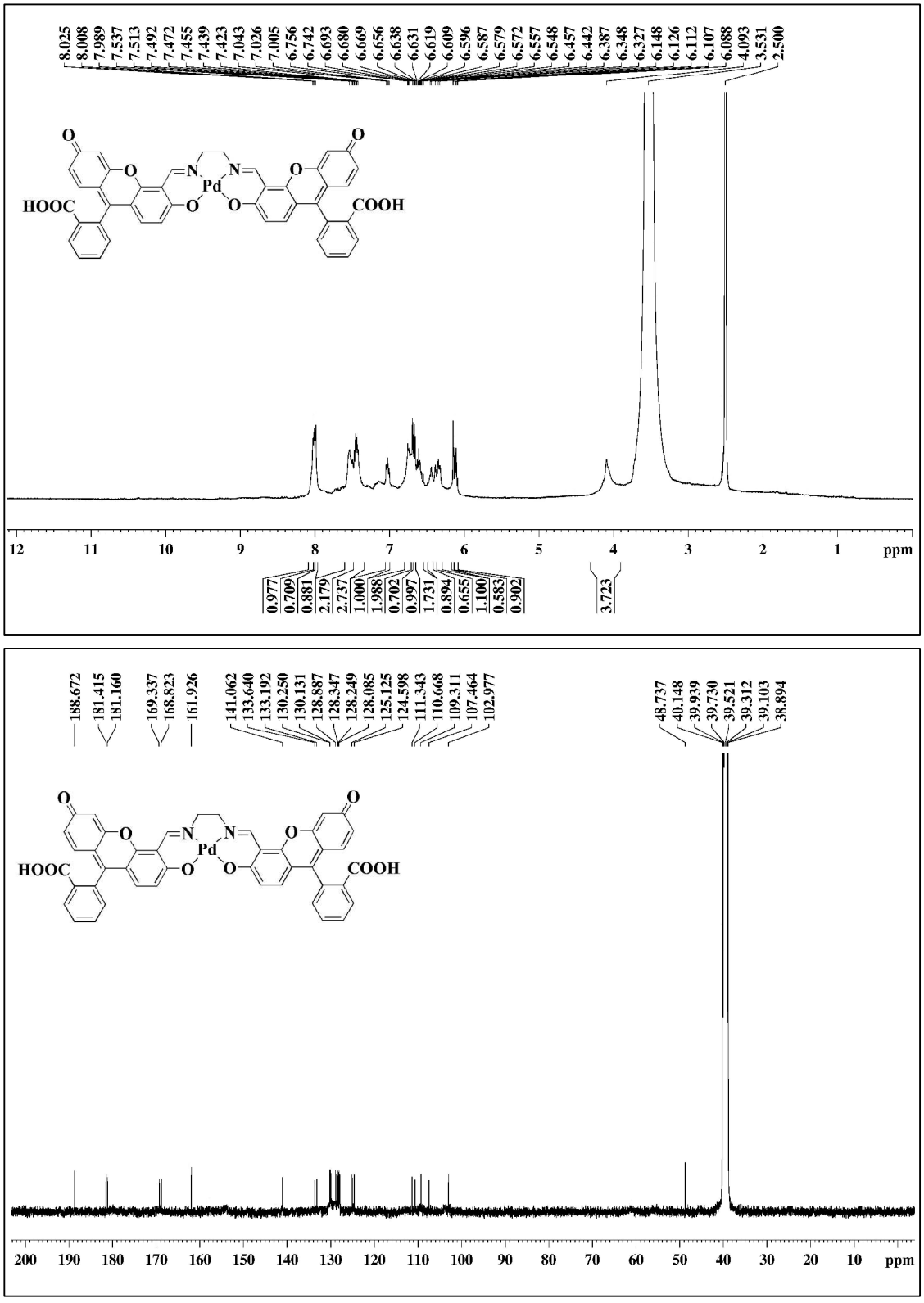

Figure S13. (A) ^{1}H-NMR and (B) ^{13}C-NMR of compound **2**.

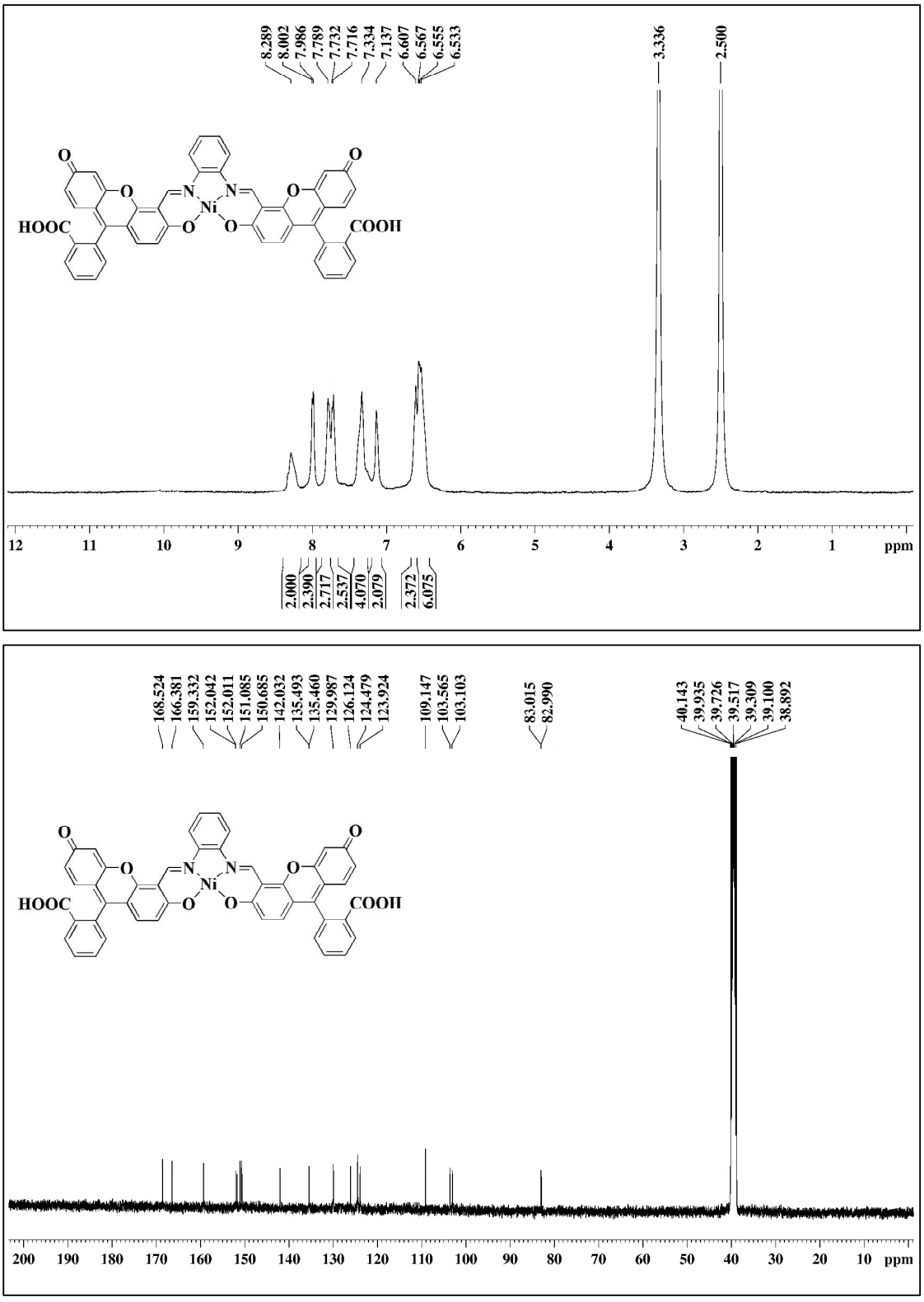

Figure S14. (A) ^{1}H-NMR and (B) ^{13}C-NMR of compound **3**.

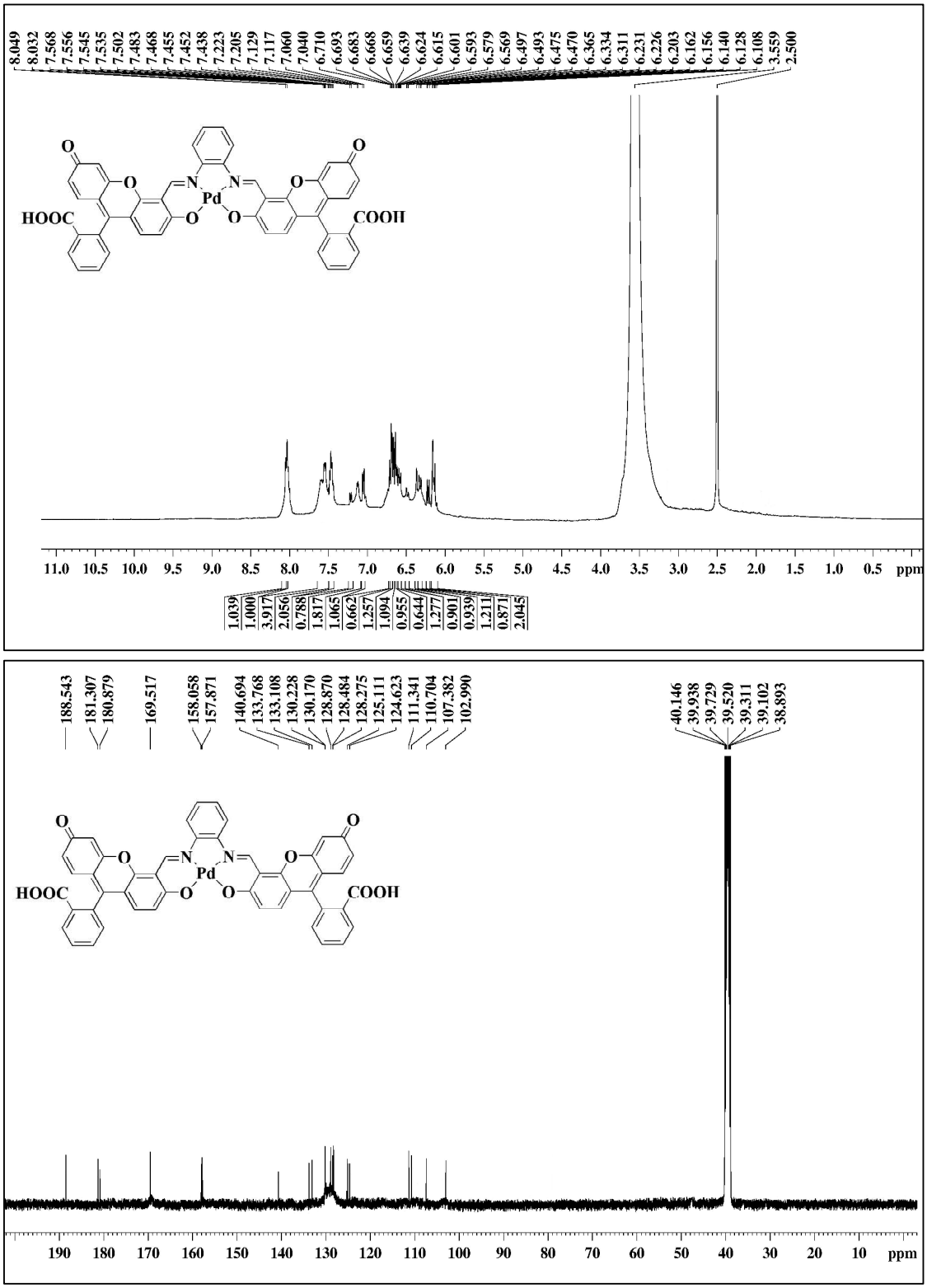

Figure S15. (A) ^{1}H-NMR and (B) ^{13}C-NMR of compound **4**.