Design, Synthesis and Biological Evaluation of Newly Synthesized Cinnamide Fluorinated Containing Compounds as Bioactive Anticancer Agents

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Supporting Information

The supporting information are divided into two parts;

1. **Spectral data (^{1}H-NMR and ^{13}C-NMR) of the synthesized compounds (pages S1-S30)**
2. **Biological Studies (pages S31-S32)**

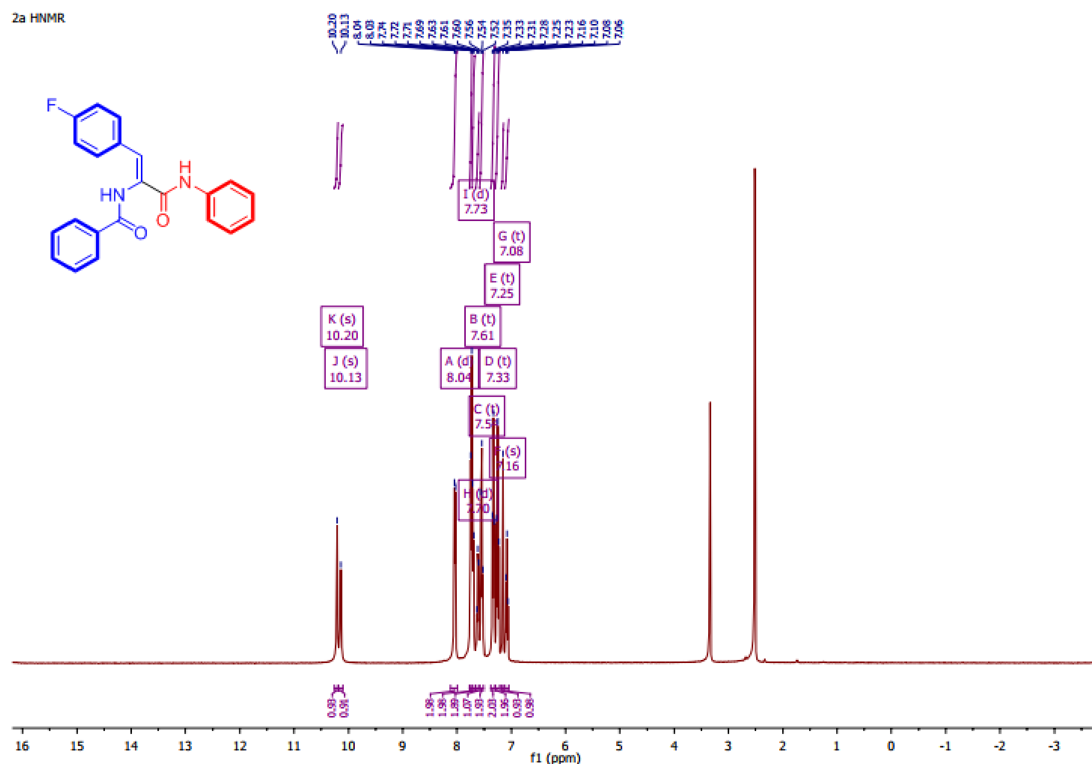

Figure S1: ^{1}H-NMR spectrum of compound **2a**

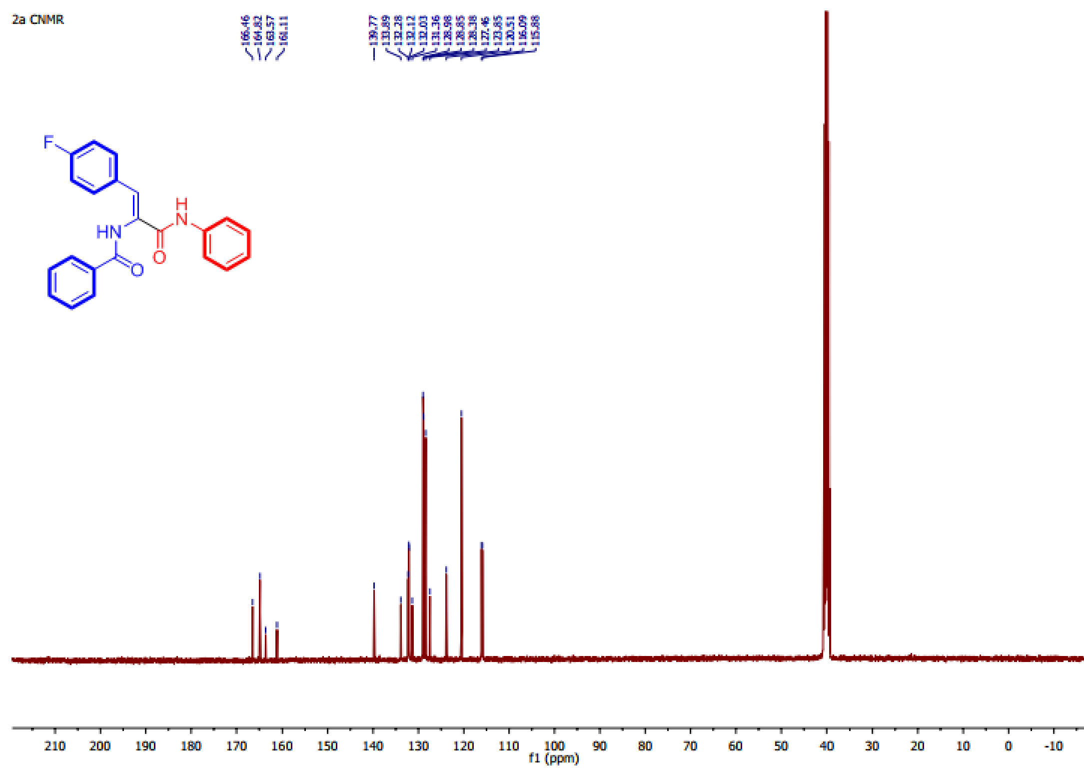

Figure S2: ^{13}C-NMR spectrum of compound **2a**

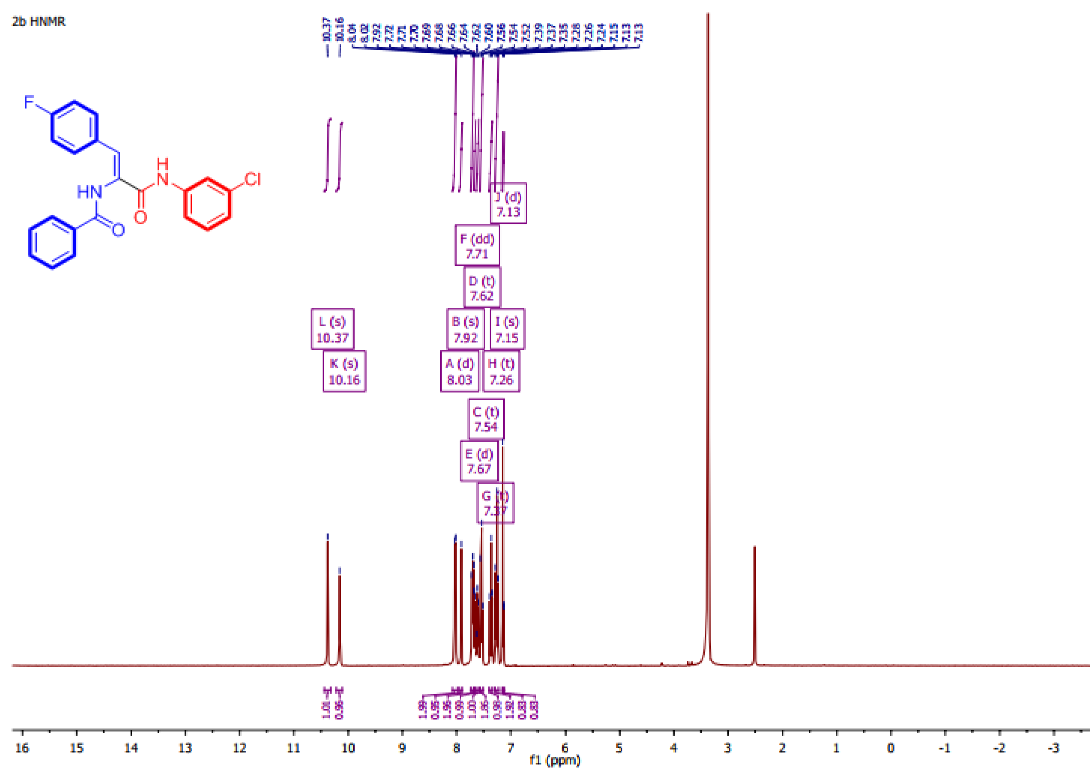

Figure S3: ¹H-NMR spectrum of compound **2b**

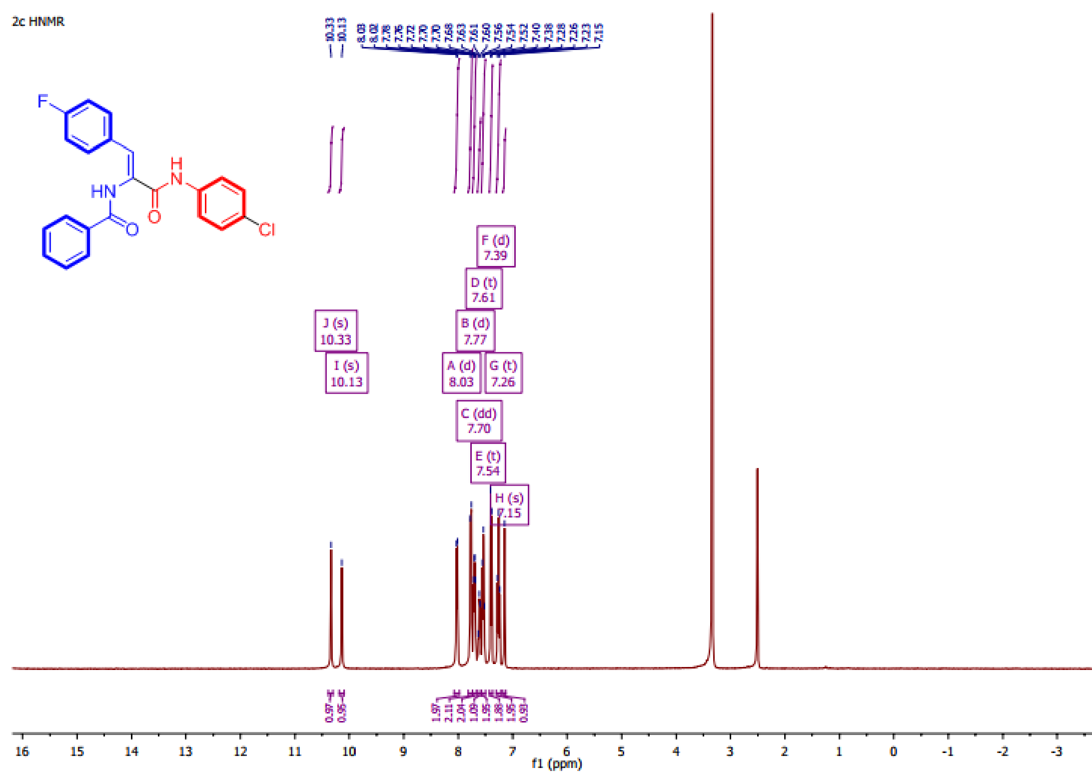

Figure S4: ^{1}H-NMR spectrum of compound **2c**

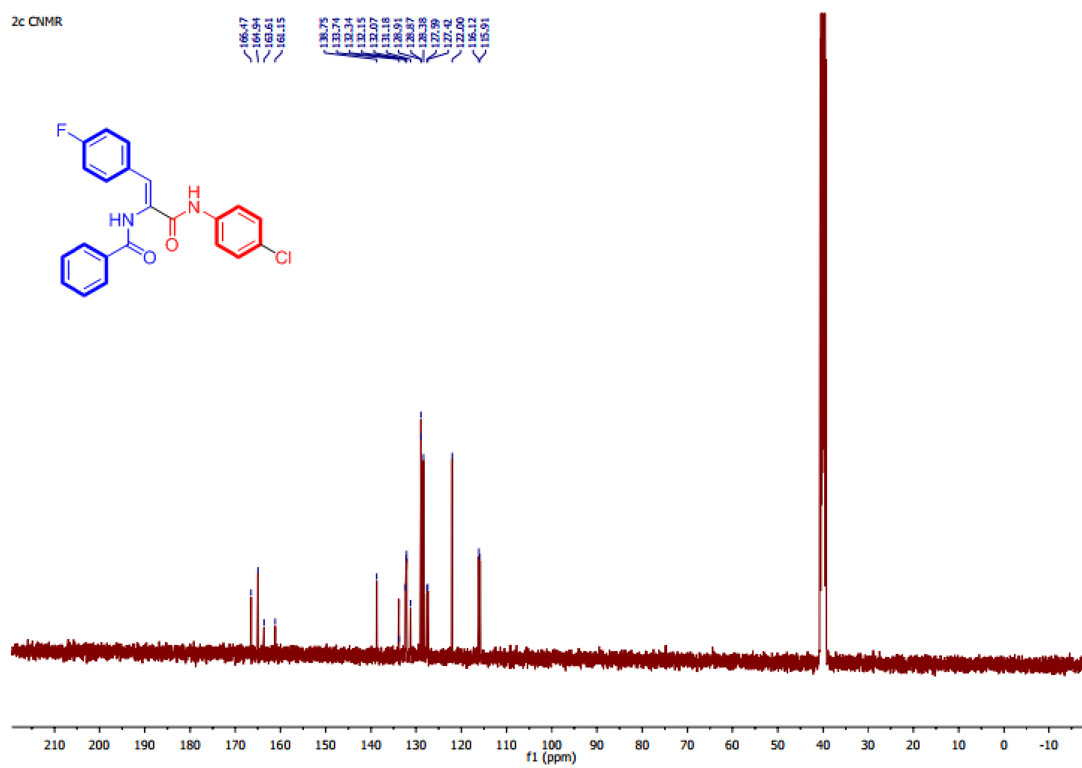

Figure S5: ^{13}C-NMR spectrum of compound **2c**

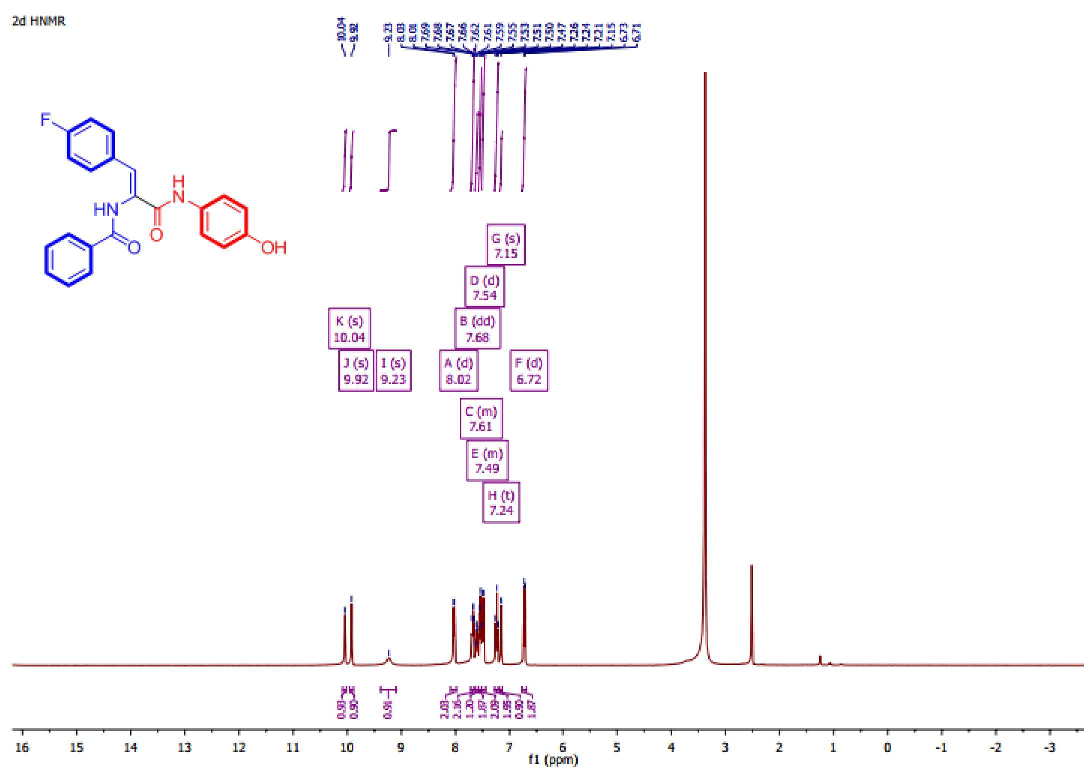

Figure S6: ^{1}H-NMR spectrum of compound **2d**

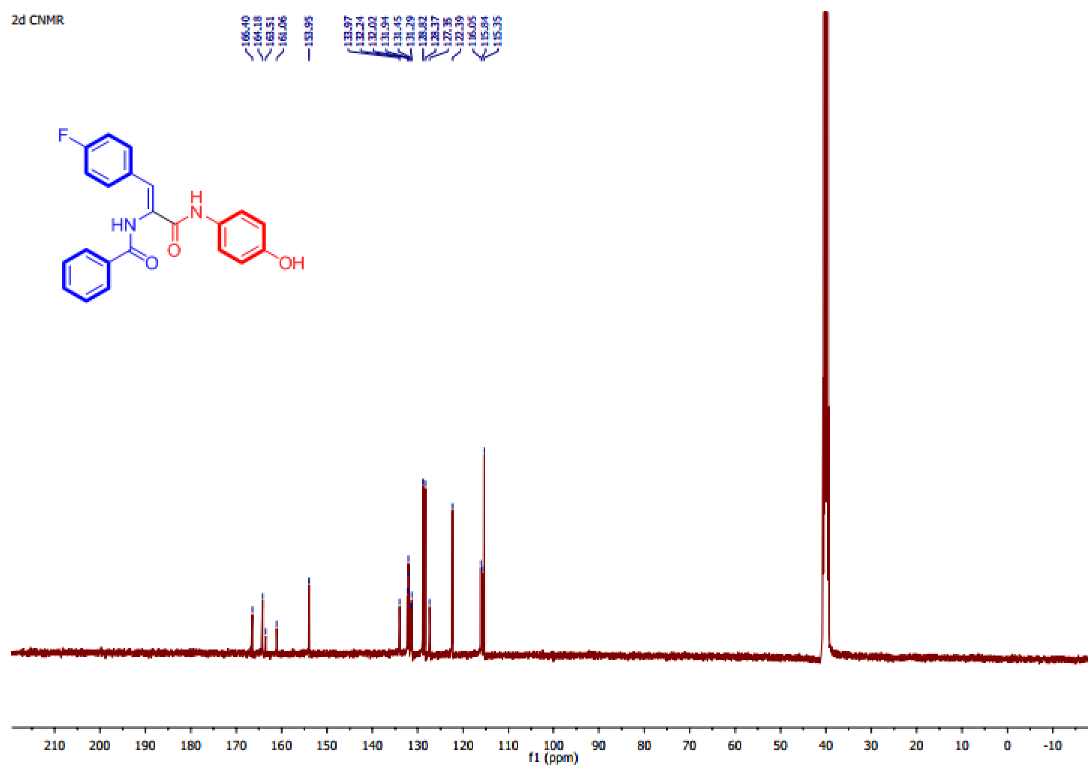

Figure S7: ^{13}C-NMR spectrum of compound **2d**

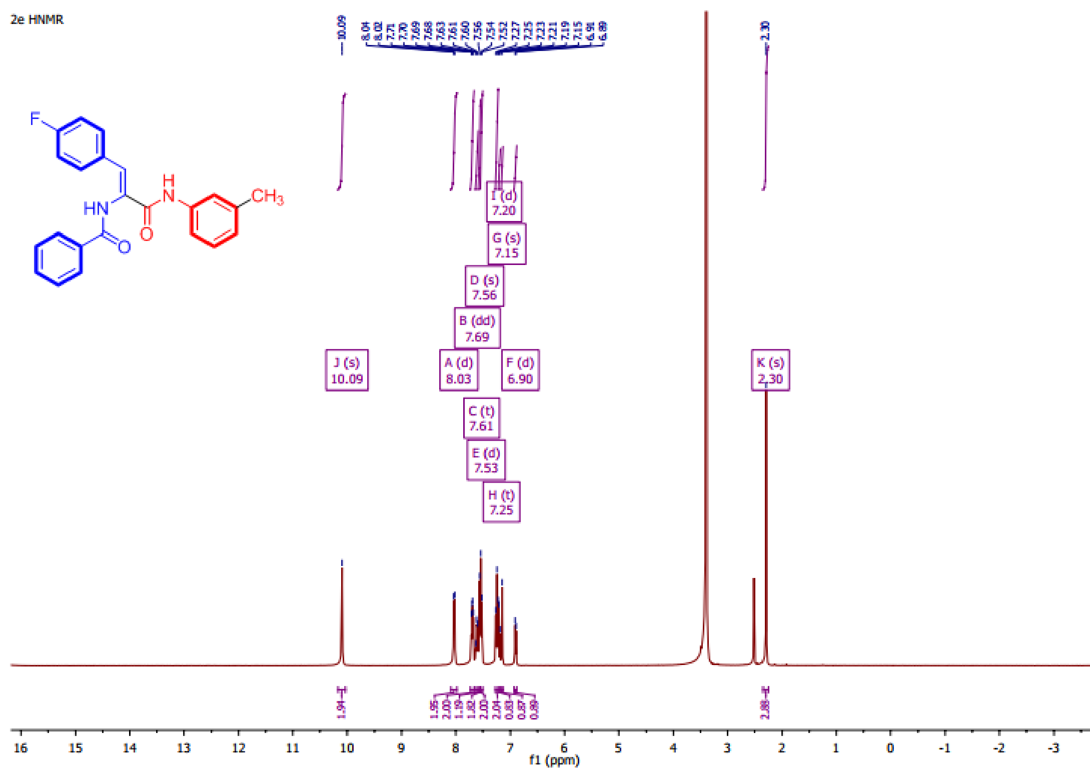

Figure S8: ^{1}H-NMR spectrum of compound **2e**

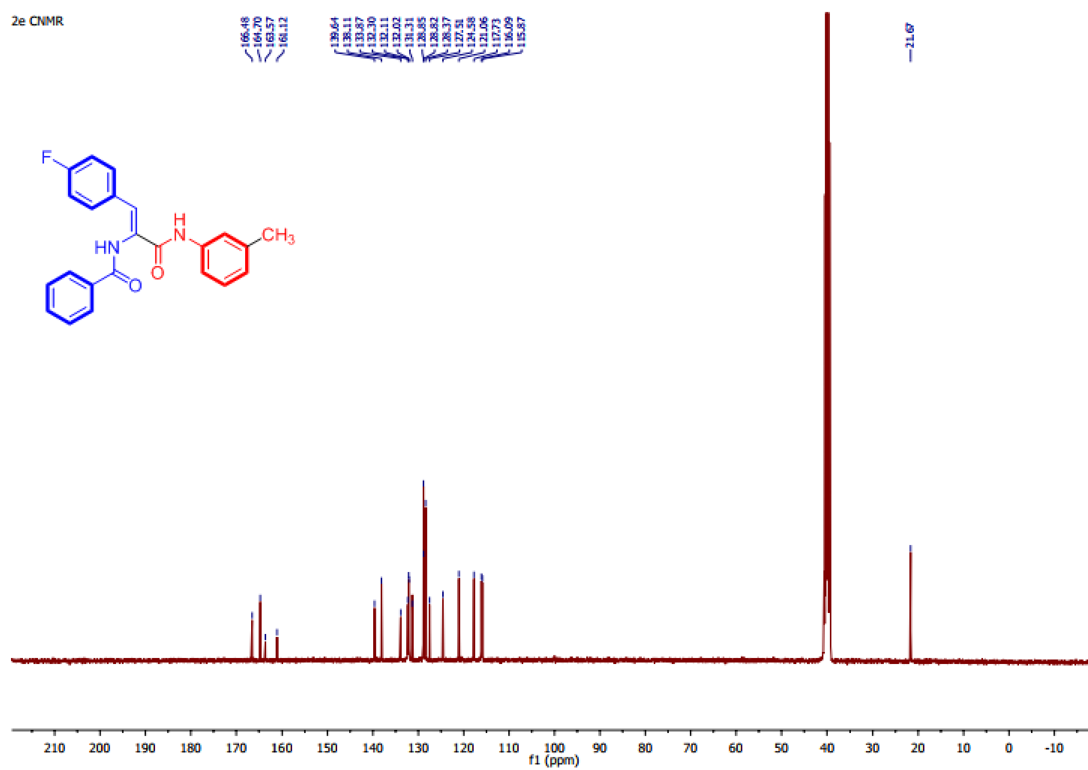

Figure S9: ^{13}C-NMR spectrum of compound **2e**

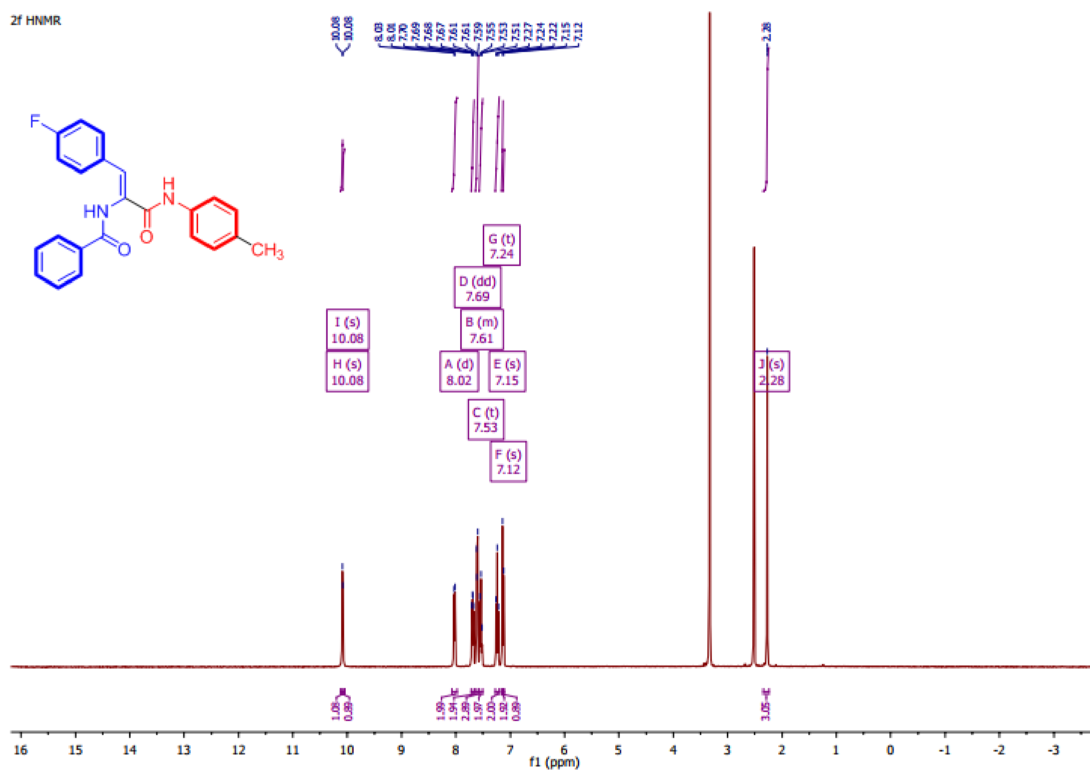

Figure S10: ^{1}H-NMR spectrum of compound **2f**

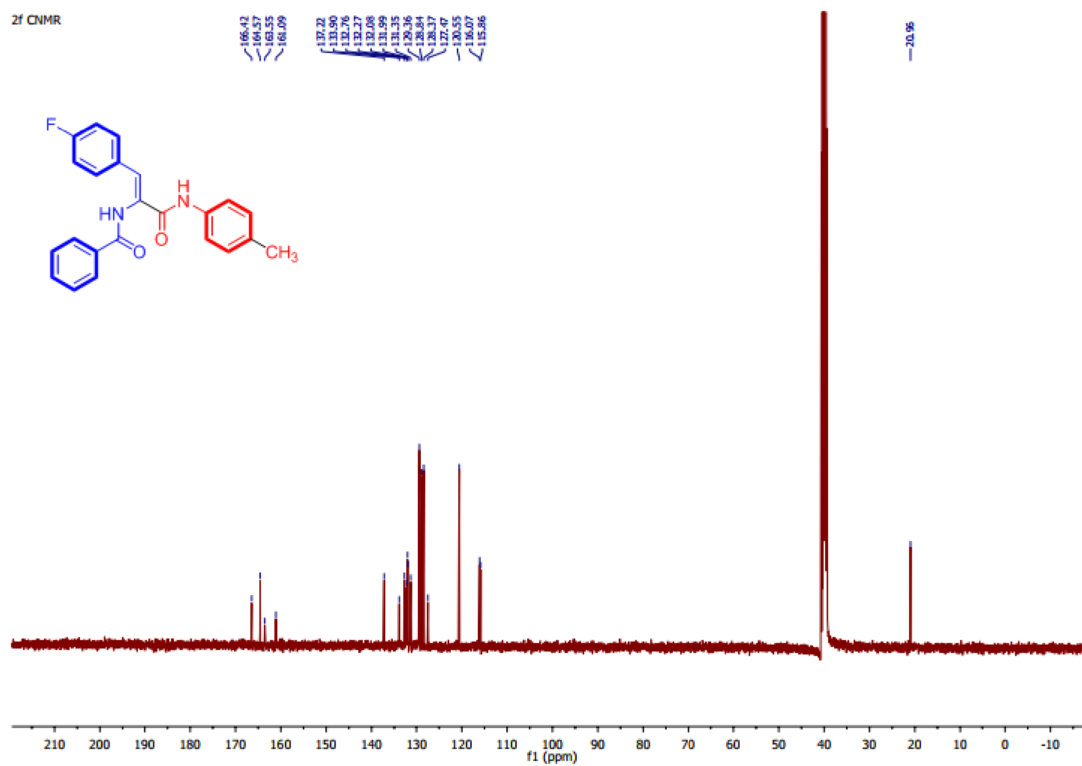

Figure S11: ^{13}C-NMR spectrum of compound **2f**

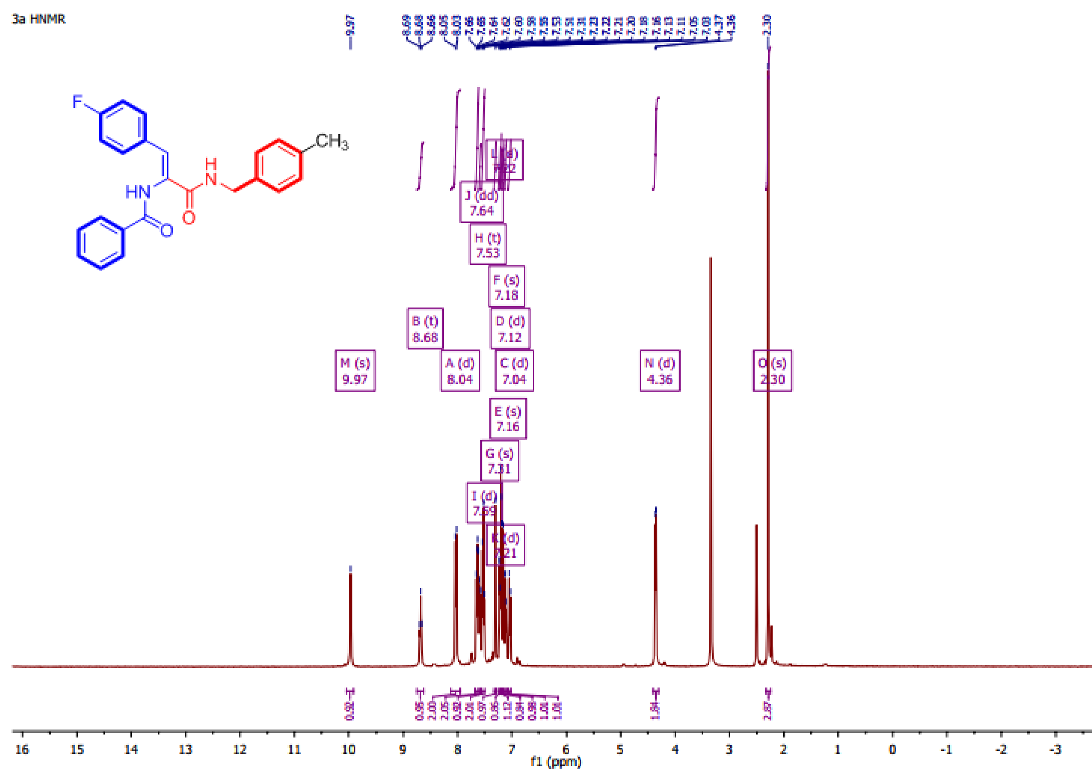

Figure S12: ^{1}H-NMR spectrum of compound **3a**

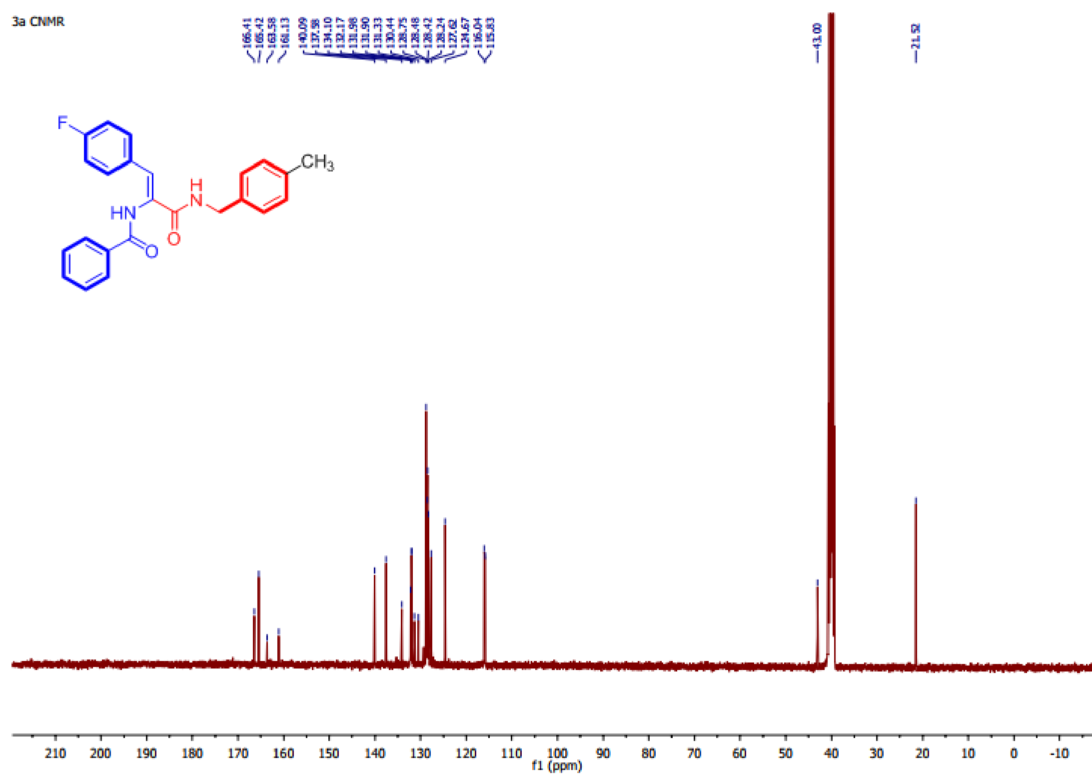

Figure S13: ^{13}C-NMR spectrum of compound **3a**

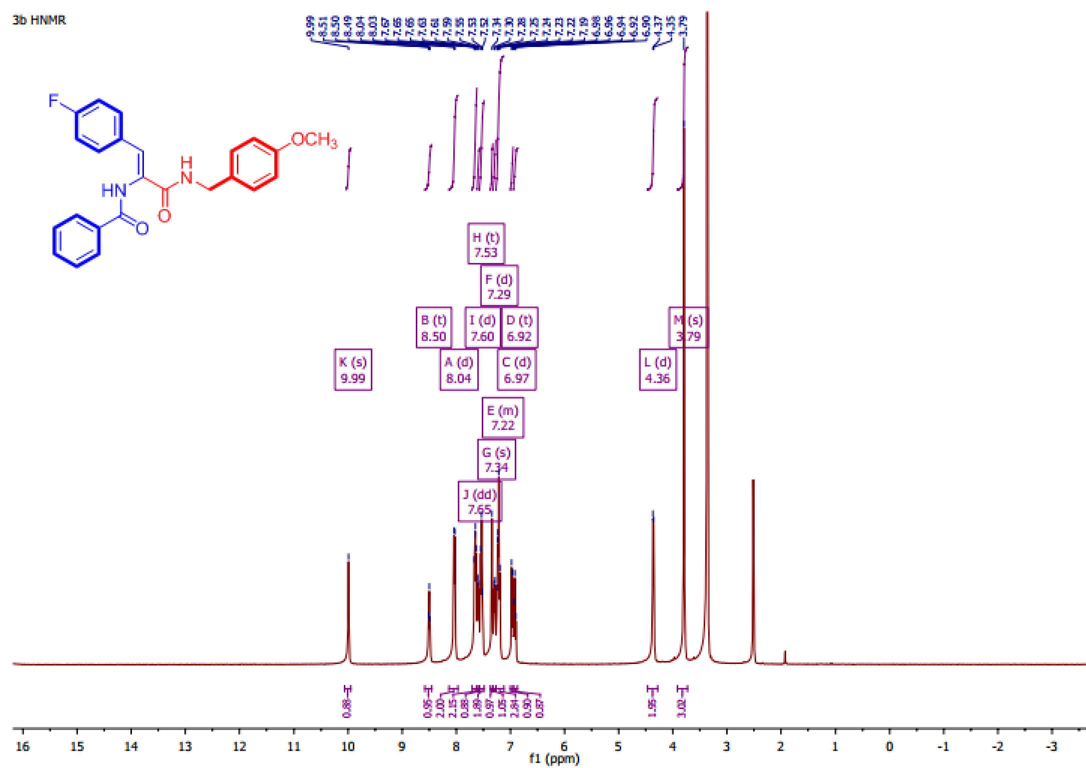

Figure S14: ^{1}H-NMR spectrum of compound **3b**

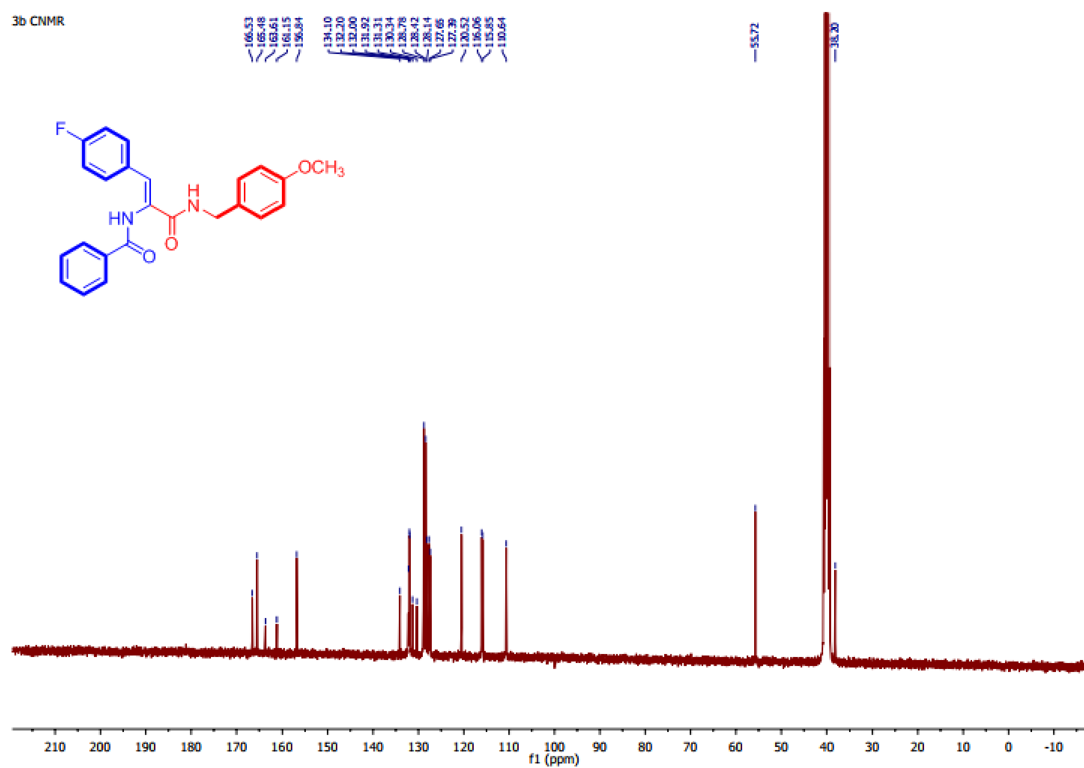

Figure S15: ^{13}C-NMR spectrum of compound **3b**

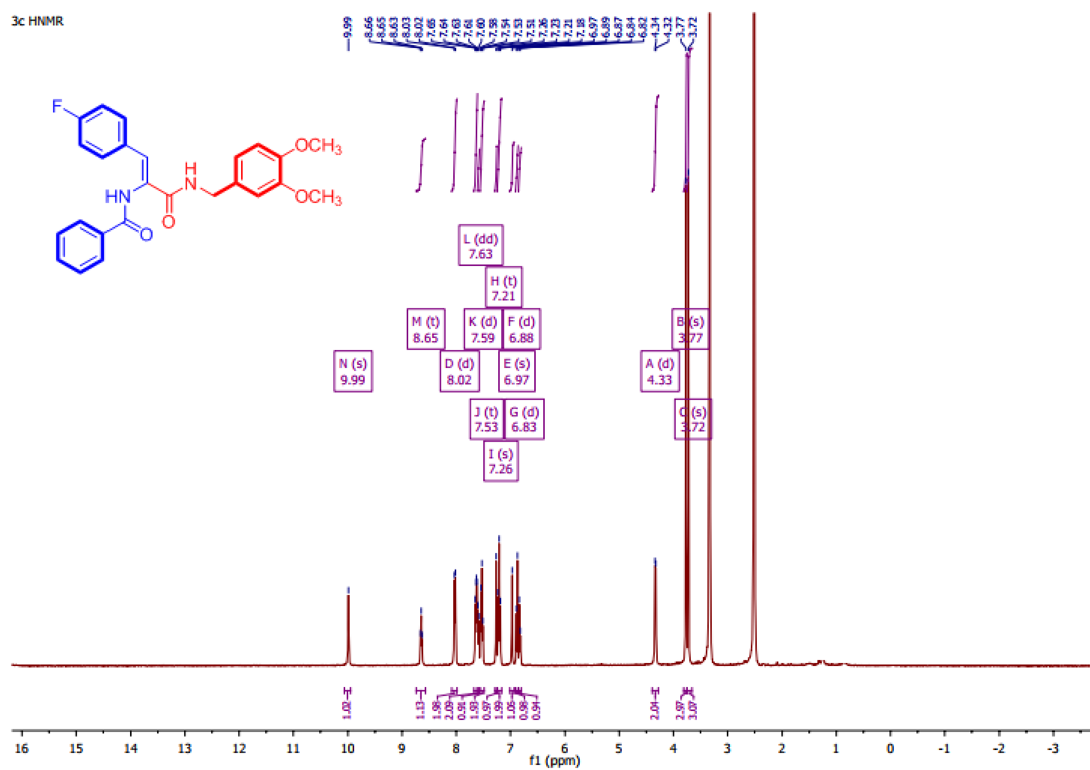

Figure S16: ^{1}H-NMR spectrum of compound **3c**

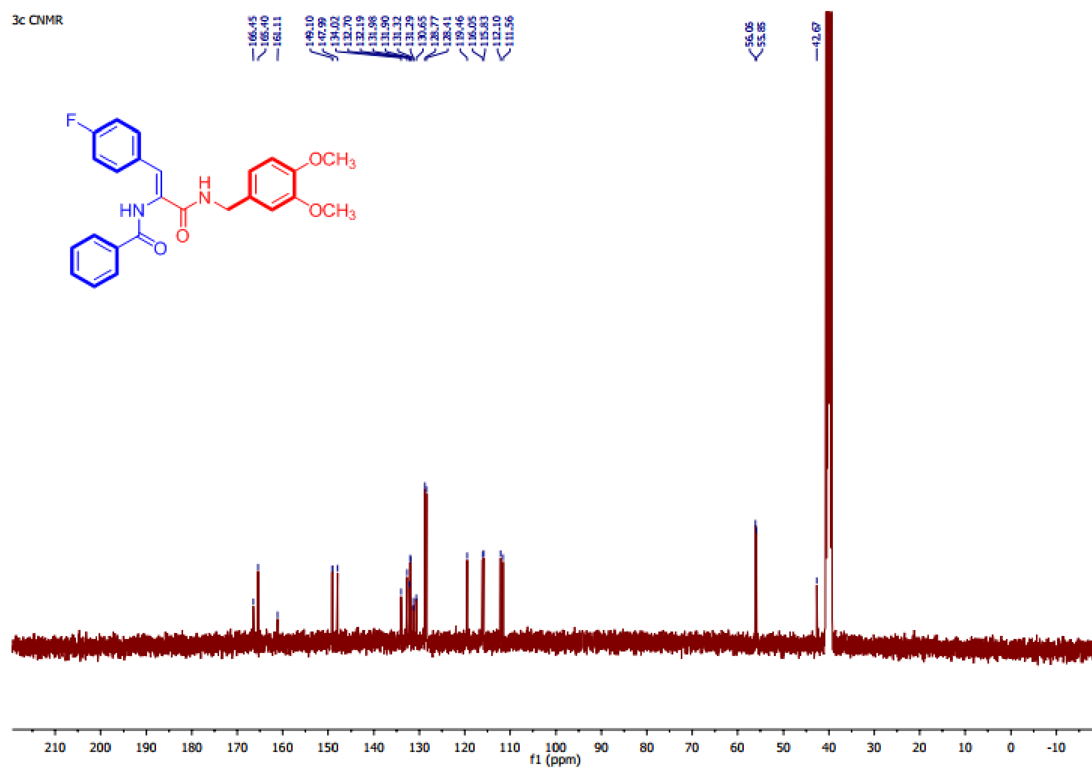

Figure S17: ^{13}C-NMR spectrum of compound **3c**

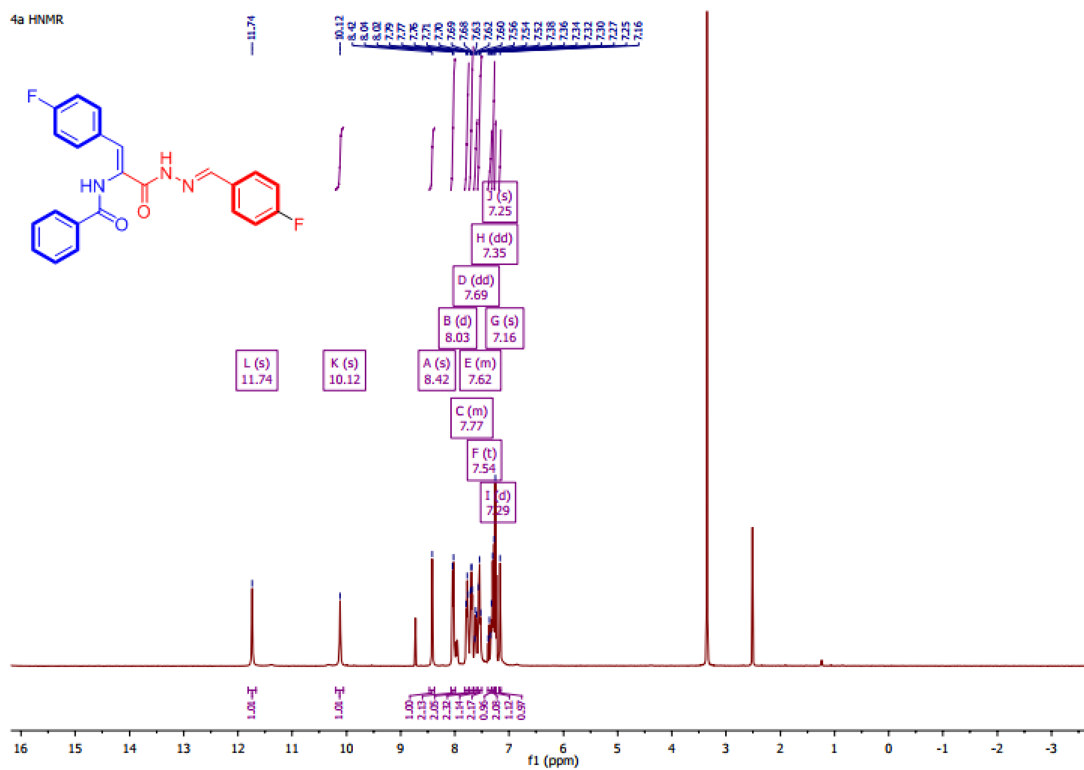

Figure S18: ^{1}H-NMR spectrum of compound **4a**

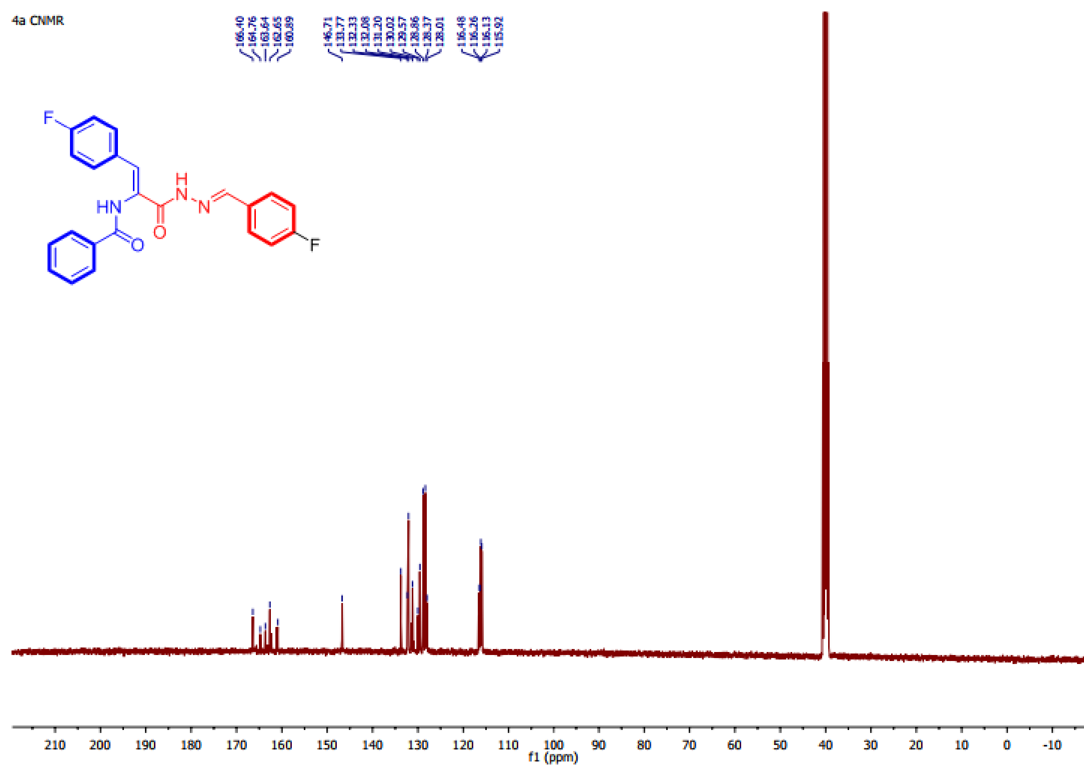

Figure S19: ^{13}C-NMR spectrum of compound **4a**

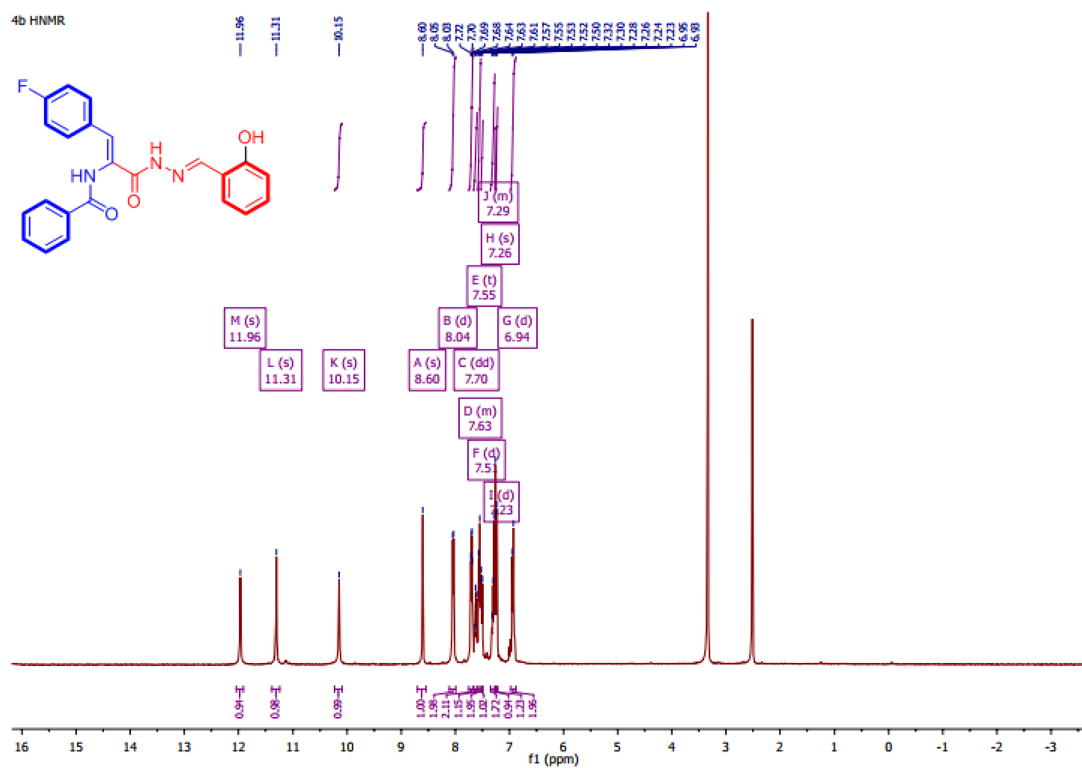

Figure S20: ^{1}H-NMR spectrum of compound **4b**

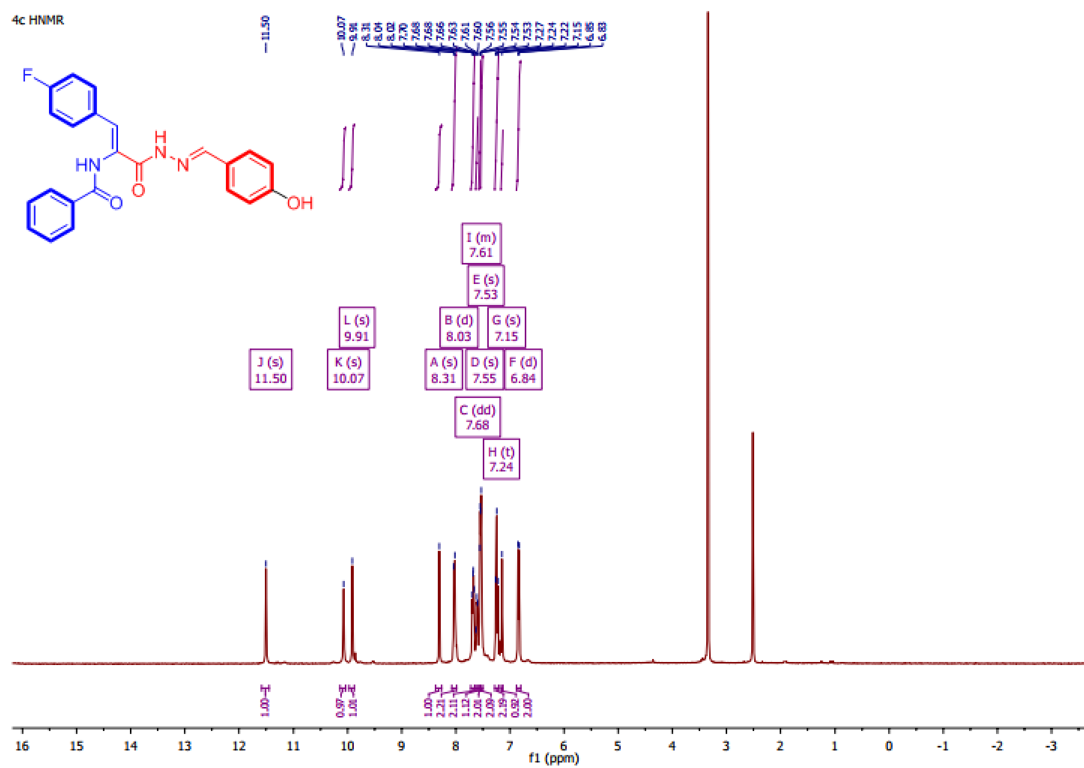

Figure S21: ^{1}H-NMR spectrum of compound **4c**

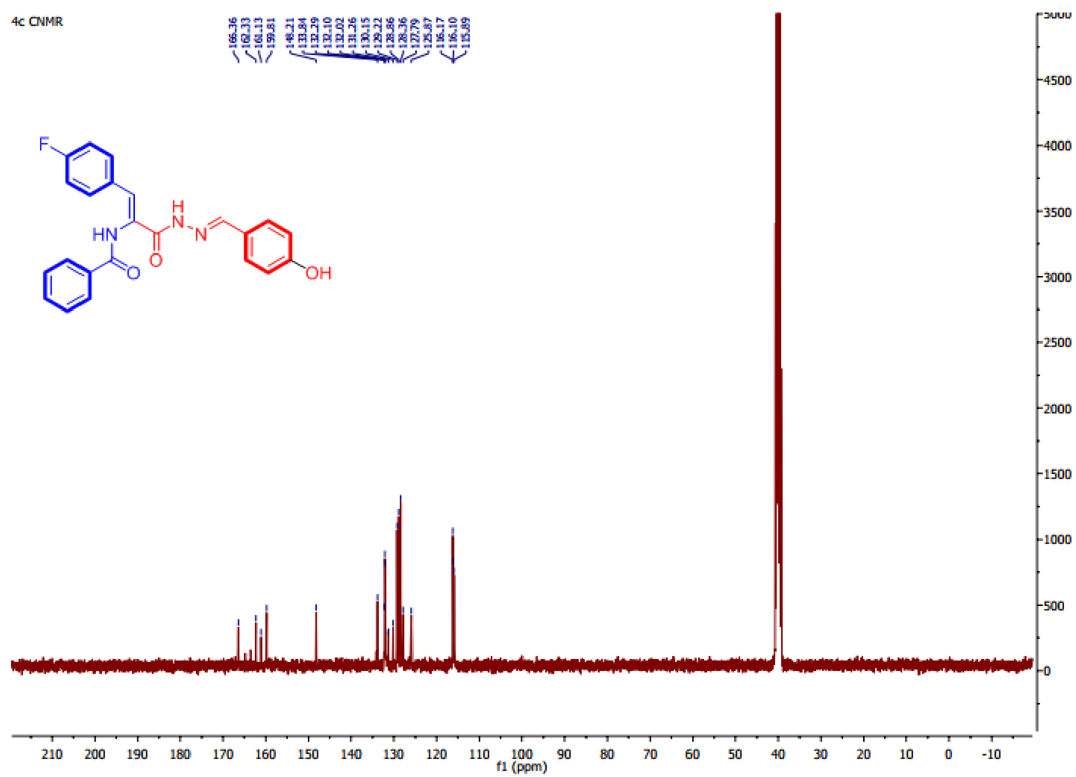

Figure S22: ^{13}C-NMR spectrum of compound **4c**

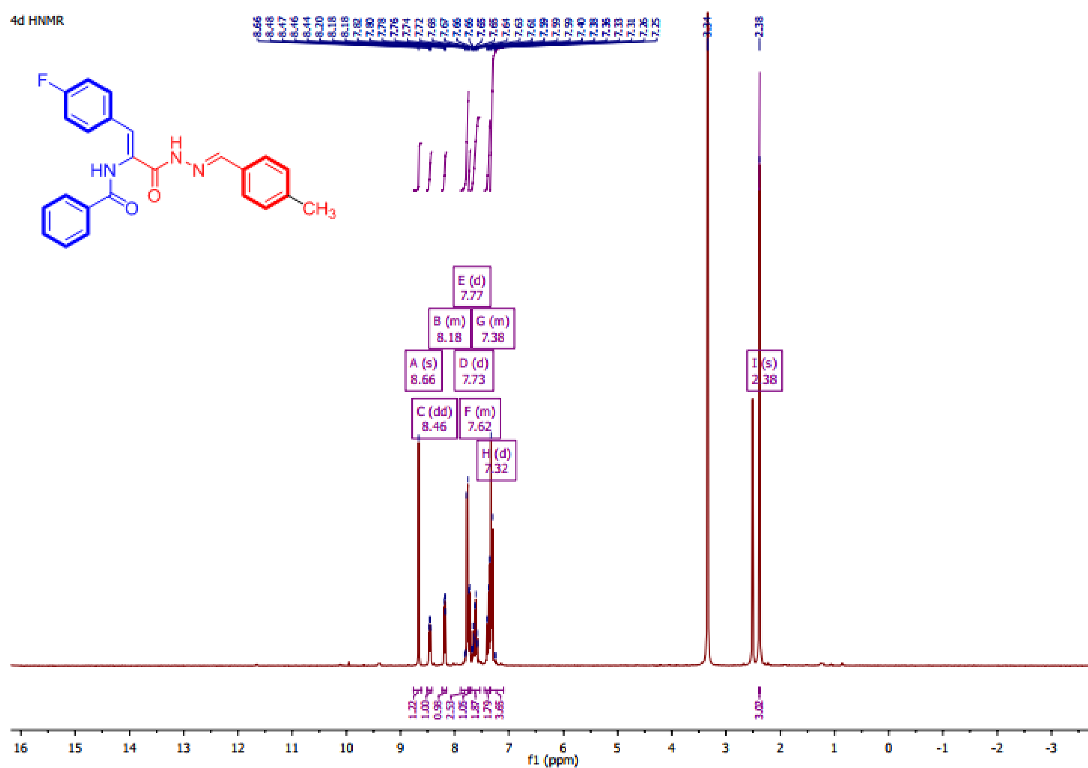

Figure S23: ^{1}H-NMR spectrum of compound **4d**

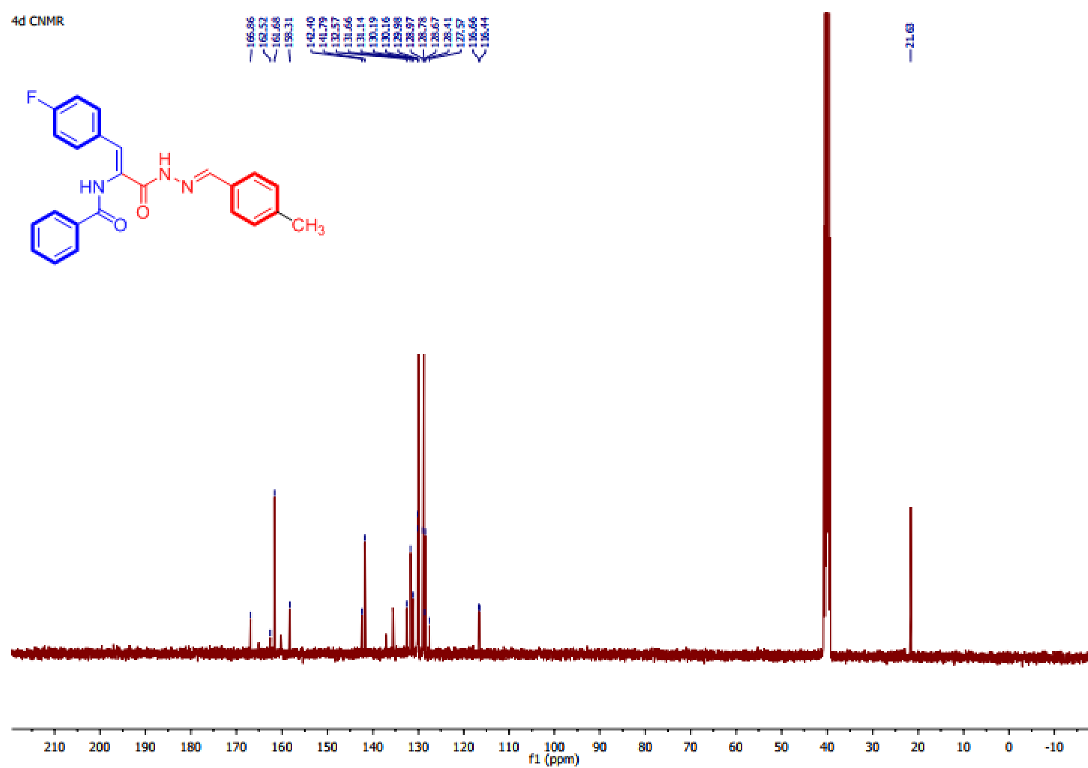

Figure S24: ^{13}C-NMR spectrum of compound **4d**

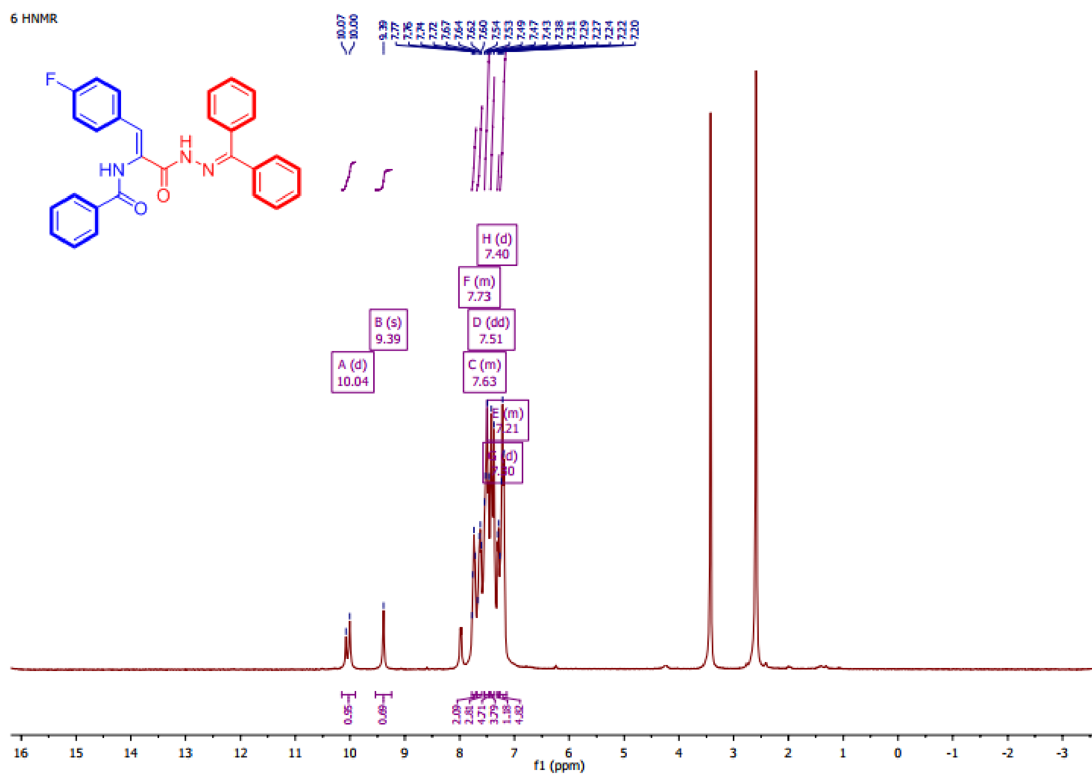

Figure S25: ^{1}H-NMR spectrum of compound **5**

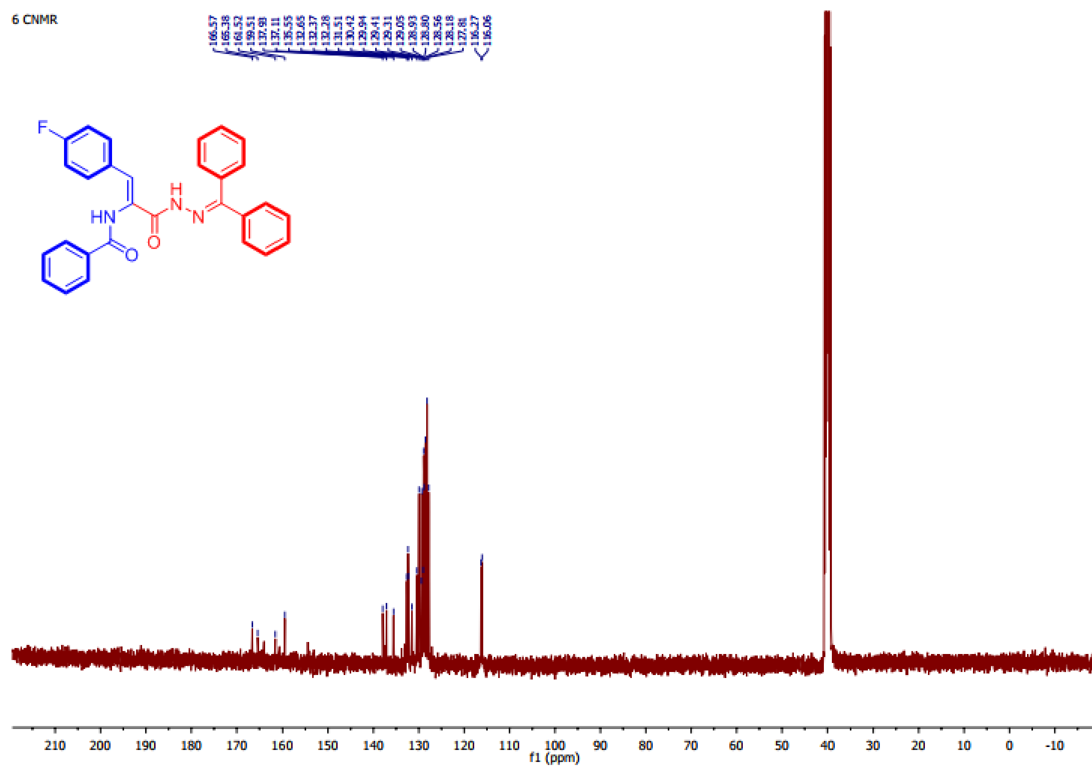

Figure S26: ^{13}C-NMR spectrum of compound **5**

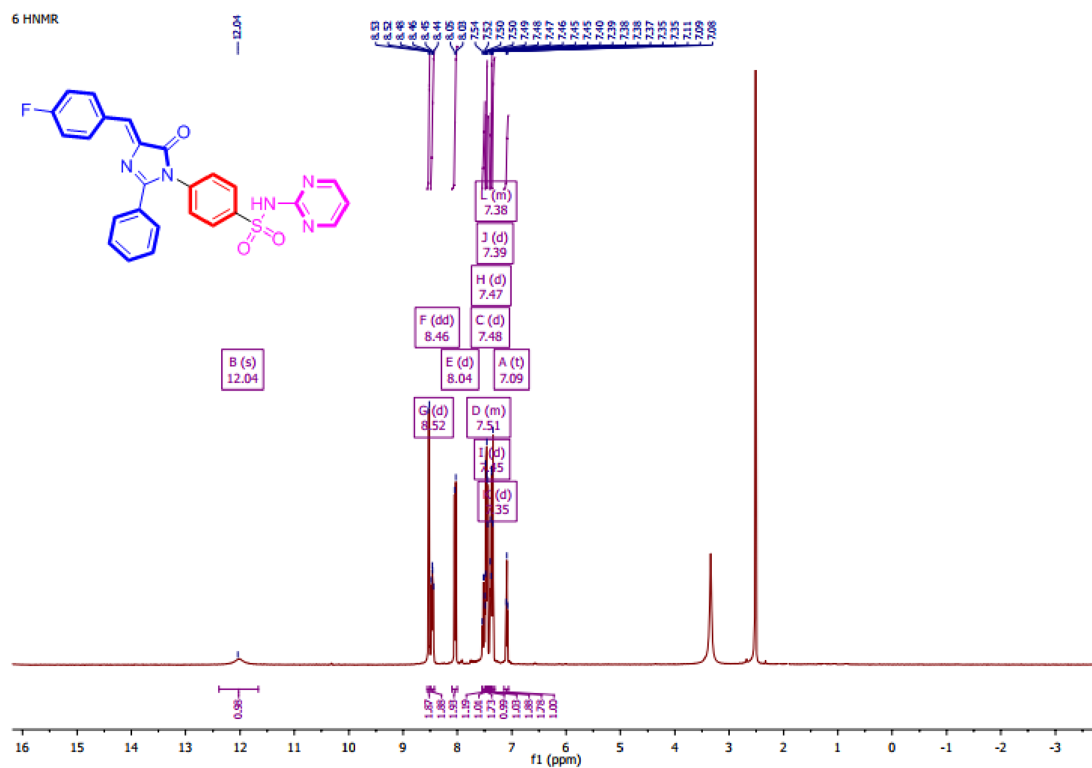

Figure S27: ^{1}H-NMR spectrum of compound **6**

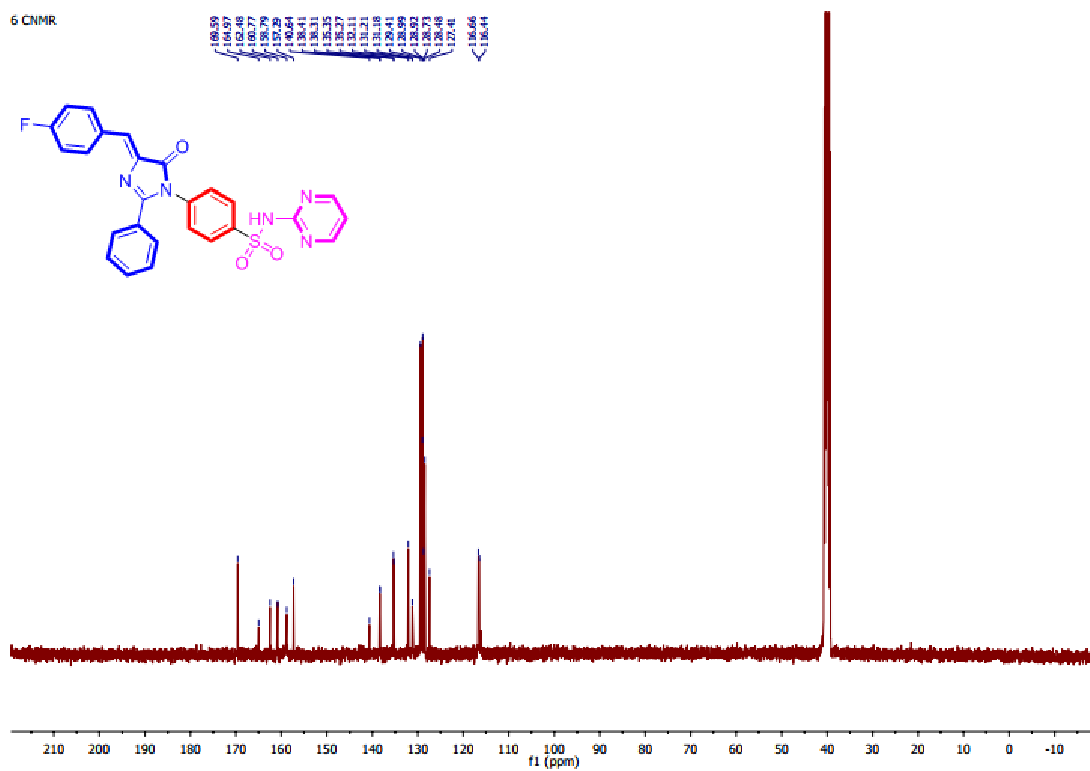

Figure S28: ^{13}C-NMR spectrum of compound **6**

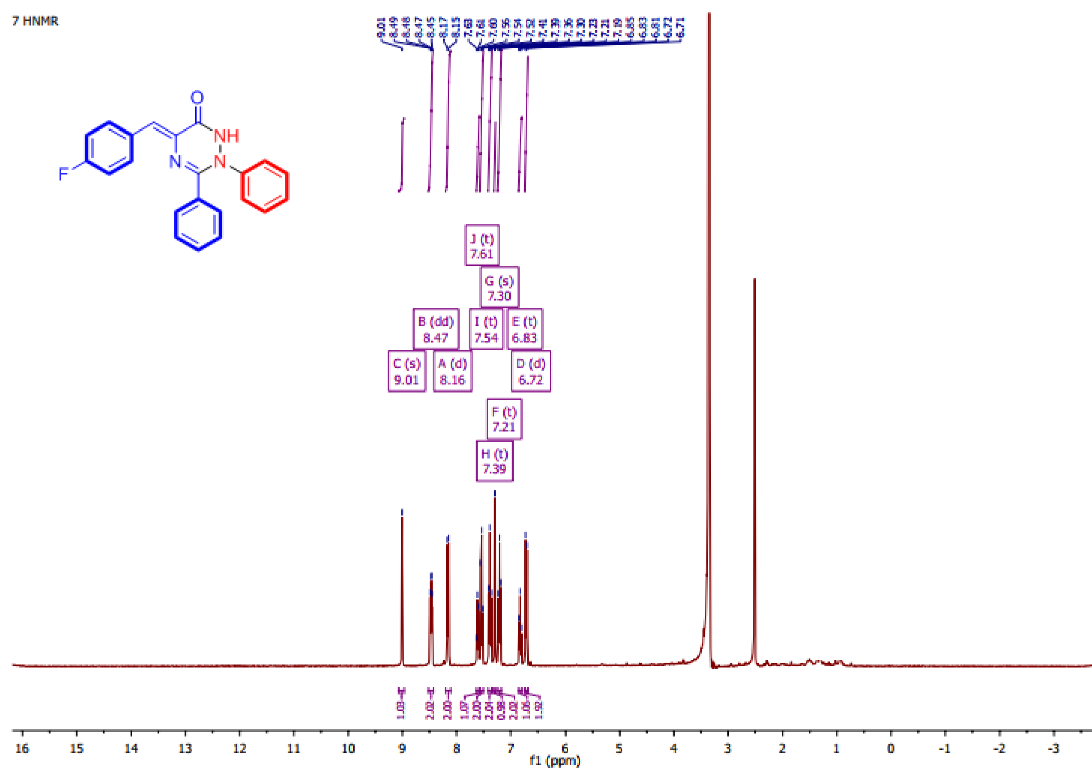

Figure S29: ^{1}H-NMR spectrum of compound **7**

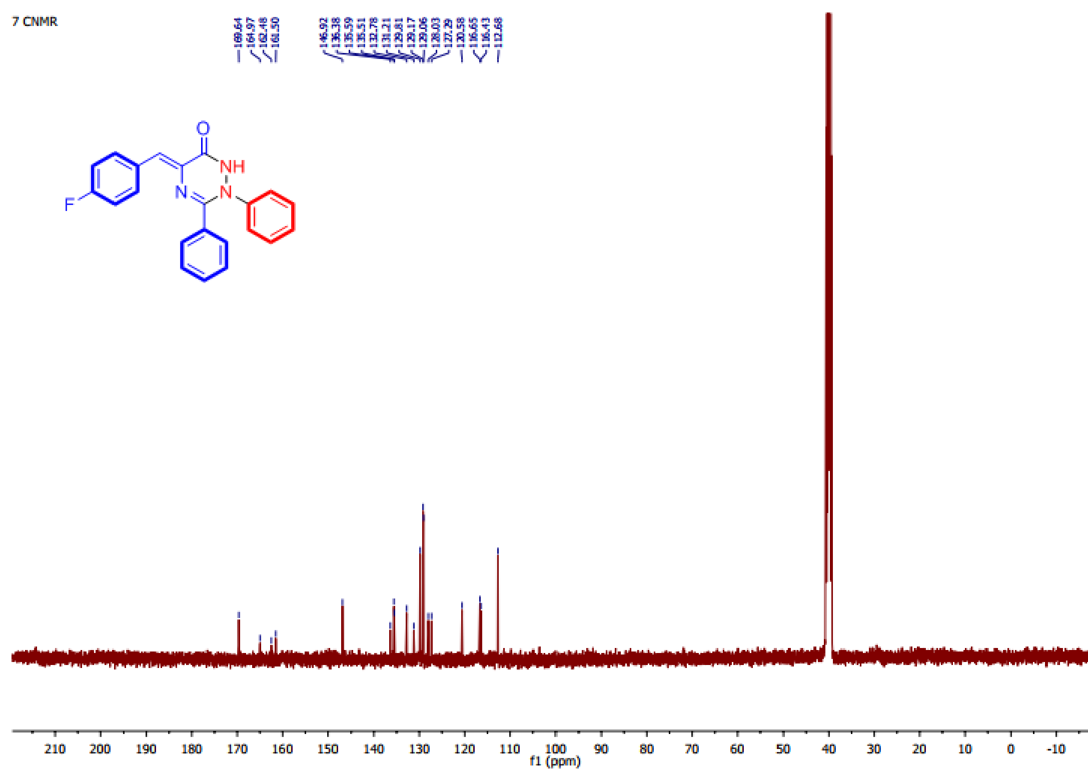

Figure S30: ^{13}C-NMR spectrum of compound **7**

2. Biological Studies

Appendix A

S4.2. Biological Studies

S4.2.1. Cytotoxic activity evaluation

To measure the cytotoxic actvity of the synthesized cinnamide derivatives **2a-7** in liver (HepG2) cell line. Cell viability assay was assessed using MTT assay method. Cells at density of 1 x 10^4 were seeded in a 96-well plate at 37 ºC for 24 h under 5% CO_2. After incubation, the cells were treated with different concentrations of the test quinoline derivatives **2a-7** and incubated for 24 h, then 20 µl of MTT solution at 5 mg/mL was applied and incubated for 4 h at 37 °C. Dimethyl sulphoxide (DMSO) in volume of 100 µl was added to each well to dissolve the purple formazan that had formed. The color intensity of the formazan product, which represents the growth condition of the cells, is quantified by using an ELISA plate reader (EXL 800, USA) at 570 nm absorbance. The experimental conditions were carried out with at least three replicates, and the experiments were repeated at least three times.

S4.2.2. EGFR kinase Assay

Compound **6** and Lapatinib were evaluated for their EGFR kinase inhibitory activity according to manufacturer's instructions using # BPS Bioscience *EGFR Kinase Assay Kit* Catalog # 40321.

S4.2.3. Cell cycle analysis of compound 6

Cell cycle analysis in HepG2 cells was investigated using fluorescent Annexin V-FITC/ PI detection kit (*BioVision* EZCell™ Cell Cycle Analysis Kit Catalog #K920) by flow cytometry assay. HepG2 cells at a density of 2×10^5 per well were harvested and washed twice in PBS. After that, the cells were incubated at 37 °C and 5% CO_2. The medium was incubated with the tested compound **6** at the IC_{50} (µM) for 48 h, washed twice in PBS, fixed with 70% ethanol, rinsed again with PBS. Afterward, medium was stained with DNA fluorochrome PI for 15 min at 37 °C. The samples were immediately analyzed using Facs Calibur flow cytometer (Becton and Dickinson, Heidelberg, Germany).

S4.2.4. Apoptosis assay for compound 6

Apoptosis in HepG2 cells was investigated using fluorescent Annexin V-FITC/ PI detection kit (*BioVision* Annexin V-FITC Apoptosis Detection Kit, Catalog #: K101) by flow cytometry assay. HepG2 cells at a density of 2×10^5 per well were treated with compound **6** at the IC_{50} (μM) for 48 h, then the cells were harvested and stained with Annexin V-FITC/ PI dye for 15 min in the dark at 37 °C. The samples were immediately analyzed using *FACS Calibur* flow cytometer (Becton and Dickinson, Heidelberg, Germany).

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Data Sheet

EGFR Kinase Assay Kit

Catalog # 40321

DESCRIPTION: The epidermal growth factor receptor (EGFR; ErbB-1; HER1) is the cell-surface receptor for members of the epidermal growth factor family. Overexpression and/or hyperactivation of EGFR kinase is associated with several human cancers such as lung, glioblastoma, and epithelian tumors of the neck and head, leading to the development of anticancer therapeutics targeting EGFR. The *EGFR Kinase Assay Kit* is designed to measure EGFR Kinase activity for screening and profiling applications using Kinase-Glo® MAX as a detection reagent. The EGFR Kinase Assay Kit comes in a convenient 96-well format, with enough purified recombinant EGFR enzyme, EGFR substrate, ATP and kinase assay buffer for 100 enzyme reactions.

COMPONENTS:

Catalog #	Reagent	Amount	Storage	
40187	EGFR (wild type)	2 μg	-80°C	***Avoid multiple freeze/ thaw cycles!***
	5x Kinase assay buffer	1.5 ml	-20°C	
	ATP (500 μM)	100 μl	-20°C	
40217	50x PTK substrate Poly(Glu:Tyr 4:1)	100 μl	-20°C	
	96-well plate, white	1	Room Temp.	

MATERIALS OR INSTRUMENTS REQUIRED BUT NOT SUPPLIED:
Kinase-Glo MAX (Promega #V6071)
Dithiothreitol (DTT, 1 M; optional)
Microplate reader capable of reading luminescence
Adjustable micropipettor and sterile tips
30°C incubator

APPLICATIONS: Useful for studying enzyme kinetics and screening small molecular inhibitors for drug discovery and HTS applications.

STABILITY: Up to 6 months when stored as recommended.

REFERENCE:

Nakamura, J.L. *Expert Opin. Ther. Targets* **11(4)**:463-472 (2007)

S4.2.5. qRT-PCR measurements of p53, Bax and Bcl2 for compound 6

Real-time PCR for p53, Bax and Bcl-2 genes expression was done using commercial Qiagen RNA extraction/ BioRad SYBER green PCR master mix according manufacturer's instructions. Briefly, 2 µl of cDNA template, 10 pMol of each forward and reverse primer, 10 µl of 2X Master Mix and to 20 µl total reaction mixture volume by nuclease free water and then was introduced to thermal cycler instrument (Thermo Scientific, USA). The cycling parameters for the PCR amplification were achieved by initial denaturation at 95 °C for 3 minutes followed by 40 cycles of 94 °C for 15 seconds and annealing/extension step at 60 °C for 1 min. Relative quantification of target genes was run on Rotor-Gene 6000 Series Software 1.7 (Build 87).

Table S1: The primer sequences for Real Time PCR assay

Gene	Sequences (5′-3′)
P53	F: AGAGTCTATAGGCCCACCCC R: GCTCGACGCTAGGATCTGAC
Bax	F: GAGGAACTGGACAGTAACATGGAGCT R: CGGCCCCAGTTGAAGTTGC
Bcl2	F: GCCGGTTCAGGTACTCAGTCATC R: GTCACCTTCACCGTTCCA
GAPDH	F: GCACCGTCAAGGCTGAGAAC R: ATGGTGGTGAAGACGCCAGT