Synthesis, Molecular modeling study, and biological evaluation of N-Acyl-anthranoylanthranilic acid Derivatives and Their Cyclized Benzoxazinones as Novel HIV-1 Nonnucleoside Reverse Transcriptase Inhibitors

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Electronic Supplementary Material

**Table S1**. 2D illustration of NNIBP-ligand interaction forces, the binding energy score ΔG, and IC50 values.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| 2D representation of the interactions | distance | Receptor- Ligand  Binding  Force | Receptor  Amino  Acid  residues | ΔG  Kcal/mole | **IC50** | **Compound** |
| G:\Hossam\nevi.png | 3.98  4.25  4.43 | H - π  H - π  H - π | Val106  Try188  Leu234 | **-7.59** | **0.11** | **Nevirapine** |
| G:\Hossam\5a.png | 4.48  3.94 | H - π  H - π | Leu100  Val106 | **-8.57** | **0.37** | **5a** |
| G:\Hossam\5b.png | 4.47  4.22 | H - π  H - π | Leu100  Trp229 | **-8.59** | **1.37** | **5b** |

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| G:\Hossam\5c.png | 4.66  4.03 | H - π  H - π | Lys101  Val106 | **-8.63** | **0.37** | **5c** |
| G:\Hossam\5d.png | 4.25 | H - π | Leu234 | **-8.95** | **1.35** | **5d** |
| G:\Hossam\5e.png | 4.25  3.25  3.82 | H - π  H - π  H - π | Leu100  Lys101  Val106 | **-9.03** | **123.52** | **5e** |

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| G:\Hossam\5f.png | 4.41  4.2 | H - π  H- π | Leu100  Trp229 | **-8.74** | **17.73** | **5f** |
| G:\Hossam\5g.png | 3.91 | H - π | Val106 | **-8.17** | **4.76** | **5g** |
| G:\Hossam\5h.png | 4.38  2.91 | H - π  H - π | Leu100  Lys101 | **-8.06** | **5.1** | **5h** |

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| G:\Hossam\5i.png | 3.94 | H - π | Val106 | **-8.47** | **2.01** | **5i** |
| G:\Hossam\5j.png | 4.3  3.87 | H - π  H - π | Leu 100  Trp229 | **-8.52** | **7.86** | **5j** |
| G:\Hossam\5k.png | 3.89 | H - π | Val106 | **-9.35** | **0.41** | **5k** |

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| G:\Hossam\5l.png | 4.4 | H - π | Lys 101 | **-8.23** | **0.24** | **5l** |
| G:\Hossam\5m.png | 2.71  3.97  3.72  4.29 | H - π  H - π  H - π  H - π | Lys101  Val106  Tyr188  Trp229 | **-8.23** | **3.07** | **5m** |
| G:\Hossam\5n.png | 3.71  3.8  3.44 | H - π  π-π  H - π | Val106  Tyr188  Trp229 | **-7.67** | **0.57** | **5n** |

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| G:\Hossam\6a.png | 4.24  4.27  3.73  3.84  3.5 | H - π  H - π  H - π  H - π  π-π | Leu100  Leu100  Val106  Val106  Tyr188 | **-8.43** | **2.37** | **6a** |
| G:\Hossam\6b.png | 3.97  3.93 | H - π  H - π | Leu100  Val106 | **-8.63** | **3.4** | **6b** |
| G:\Hossam\6c.png | 3.98  3.93 | H - π  H - π | Leu100  Val106 | **-8.69** | **2.38** | **6c** |

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| G:\Hossam\6d.png | 4.25  3.64  3.99 | H - π  H - π  π-π | Leu100  Val106  Tyr188 | **-8.74** | **0.03** | **6d** |
| G:\Hossam\6e.png | 4.19  3.68  3.82 | H - π  H - π  π-π | Leu100  Val106  Tyr188 | **-8.63** | **1.24** | **6e** |
| G:\Hossam\6f.png | 4.24  3.52 | H - π  π-π | Leu 100  Tyr188 | **-8.33** | **0.66** | **6f** |
| G:\Hossam\6g.png | 3.1  3.96 | H - π  H - π | Lys101  Leu100 | **-8.35** | **0.72** | **6g** |
| G:\Hossam\6h.png | 4.33  3.64 | H - π  H - π | Leu 100  Val106 | **-8.34** | **0.36** | **6h** |
| G:\Hossam\6i.png | 4.21  4.24  3.74  3.49 | H - π  H - π  H - π  π-π | Leu100  Leu100  Val106  Tyr188 | **-8.54** | **1.21** | **6i** |
| G:\Hossam\6j.png | 4.33  4.36  3.75 | H - π  H - π  H- π | Leu100  Lys101  Trp229 | **-8.3** | **0.43** | **6j** |
| G:\Hossam\6k.png | 3.95  3.71 | H - π  H - π | Leu100  Val106 | **-8.89** | **0.44** | **6k** |
| G:\Hossam\6l.png | 4.17  3.77 | H - π  H - Cl | Lys101  Lys172 | **-8.19** | **0.08** | **6l** |

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| G:\Hossam\6m.png | 3.68 | π- π | Tyr188 | **-7.67** | **0.42** | **6m** |
| G:\Hossam\6n.png | 4.22  4.29  3.7  3.52 | H - π  H - π  H - π  π-π | Leu100  Leu100  Val106  Tyr188 | **-7.67** | **3.24** | **6n** |

Table S2 X-ray crystallographic data of compound 6a

|  |  |
| --- | --- |
| 21H14N2O3 | Empirical formula |
| 342.34 | Formula weight/g mol−1 |
| 0.080 × 0.050 × 0.030 | crystal size/mm |
| 1.415 g cm−3 | calculated density |
| 103.(2) | Temperature K |
| Triclinic | Lattice |
| 'P -1', Z=2 | space group |
| a=8.4546(5) Å, b=9.4173(6) Å,  c=10.9991(8) Å, α=67.922(2) °,  β=83.073(2) °, γ=83.382(2) °,  V=803.27(9) Å3, Z= 2 | Unit cell dimensions |
| 0.096 mm−1 | Absorption coefficient μ |
| Multi-Scan | absorption correction |
| 0.91, 1.00 | Minimum and maximum transition factor |
| 8324 | Measured reflections |
| 2501 | Observed reflections |
| 3260 | Reflections in refinement |
| 3.209–26.362 | Theta range for data collection θ range |
| Full matrix least squares on F2 | Refinement method |
| 0/239 | Restraints/ Parameters |
| 1.017 | Goodness of fit on F2(S) |
| R(Fobs)= 0.0459  Rw (F2)= 0.1131  mean σ(I)= 0.0501 I | Final R indices |
| 1.415 | calc. density/g cm−3 |
| E = 0.213 Å−3 | maximum electron density |
| E = −0.256Å−3 | minimum electron density |
| 0.0480, 0.2403 | x, y (weighting scheme) |
| H(C) constr, H(N) refall | hydrogen refinement |
| 0.001 | shift/errormax |
| 0.0379 | Rint |

**Table S3** Docked **5a-n** and **6a-n**bound tothe amino acid residues in the NNIBP, and their IC50using**nevirapine**reference

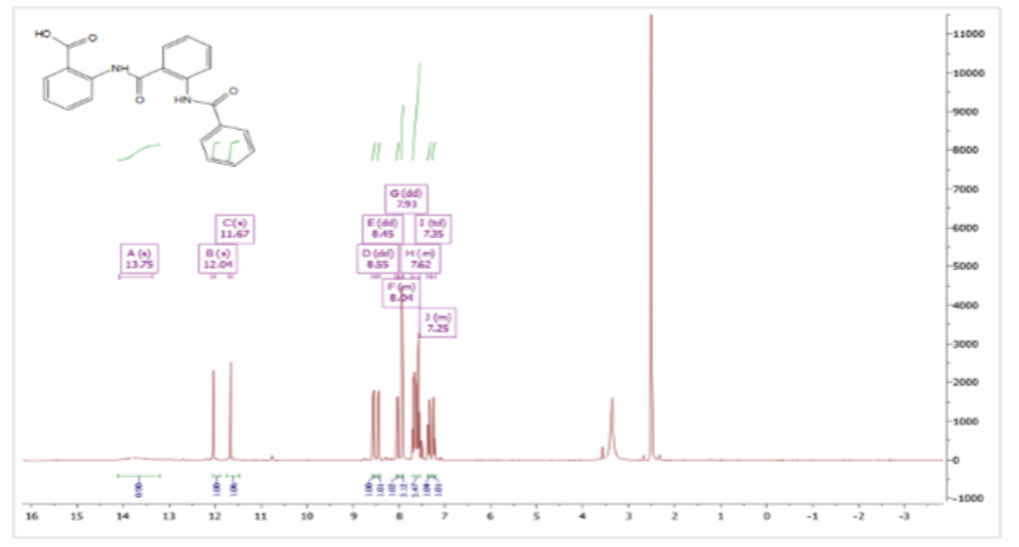
|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Entry** | Amino acid residues bound to the ligands | | | | | | | | **Ic50**  **(µM**) |
| **aLeu100**  **(L)** | **aLys101**  **(K)** | **bVal106**  **(V)** | **Lys172**  **(K)** | **bTyr188**  **(Y)** | **Trp229**  **(W)** | **Leu234**  **(L)** | **His235 (H)** |
| **Nevirapine** | **-** | **-** | **+** | **-** | **+** | **-** | **+** | **-** | **0.11** |
| **5a** | **+** | **-** | **+** | **-** | **-** | **-** | **-** | **-** | **0.37** |
| **5b** | **­+** | **-** | **-** | **­-** | **-** | **+** | **-** | **-** | **1.37** |
| **5c** | **­-** | **+** | **+** | **-** | **-** | **-** | **-** | **-** | **0.37** |
| **5d** | **-** | **-** | **-** |  | **-** | **-** | **+** | **-** | **1.35** |
| **5e** | **+** | **+** | **+** | **-** | **-** | **-** | **-** | **-** | **123.5** |
| **5f** | **+** | **-** | **-** | **-** | **-** | **+** | **-** | **-** | **17.73** |
| **5g** | **-** | **-** | **+** | **-** | **-** | **-** | **-** | **-** | **4.76** |
| **5h** | **+** | **+** | **-** | **-** | **-** | **-** | **-** | **-** | **5.10** |
| **5i** | **-** | **-** | **+** | **-** | **-** | **-** | **-** | **-** | **2.01** |
| **5j** | **+** | **-** | **-** | **-** | **-** | **+** | **-** | **-** | **7.86** |
| **5k** | **-** | **-** | **+** | **-** | **-** | **-** | **-** | **-** | **0.41** |
| **5l** | **-** | **+** | **-** | **-** | **-** | **-** | **-** | **-** | **0.24** |
| **5m** | **-** | **+** | **+** | **-** | **+** | **+** | **-** | **-** | **3.07** |
| **5n** | **-** | **-** | **+** | **-** | **+** | **+** | **-** | **-** | **0.57** |
| **6a** | **++** | **-** | **++** | **-** | **+** | **-** | **-** | **-** | **2.37** |
| **6b** | **+** | **-** | **+** | **-** | **-** | **-** | **-** | **-** | **3.4** |
| **6c** | **+** | **-** | **+** | **-** | **-** | **-** | **-** | **-** | **2.38** |
| **6d** | **+** | **-** | **+** | **-** | **+** | **-** | **-** | **-** | **0.03** |
| **6e** | **+** | **-** | **+** | **-** | **+** | **-** | **-** | **-** | **1.24** |
| **6f** | **+** | **-** | **-** | **-** | **+** | **-** | **-** | **-** | **0.66** |
| **6g** | **+** | **+** | **-** | **-** | **-** | **-** | **-** | **-** | **0.72** |
| **6h** | **+** | **-** | **+** | **-** | **+** | **-** | **-** | **+** | **0.36** |
| **6i** | **++** | **-** | **+** | **-** | **+** | **-** | **-** | **-** | **1.21** |
| **6j** | **+** | **+** | **-** | **-** | **-** | **+** | **-** | **-** | **0.43** |
| **6k** | **+** | **-** | **+** | **-** | **-** | **-** | **-** | **-** | **0.44** |
| **6l** | **-** | **+** | **-** | **+** | **-** | **-** | **-** | **-** | **0.08** |
| **6m** | **-** | **-** | **-** | **-** | **+** | **-** | **-** | **-** | **0.42** |
| **6n** | **++** | **-** | **+** | **-** | **+** | **-** | **-** | **-** | **3.25** |

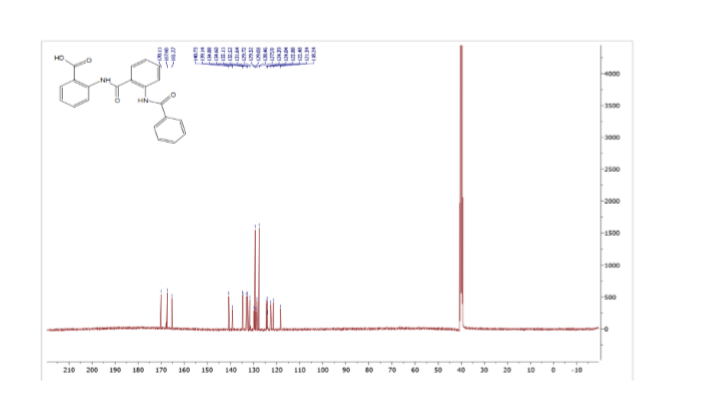
a minor mutable residue, b major mutable residues, + = one interaction, ++ = two interactions,= no interaction.

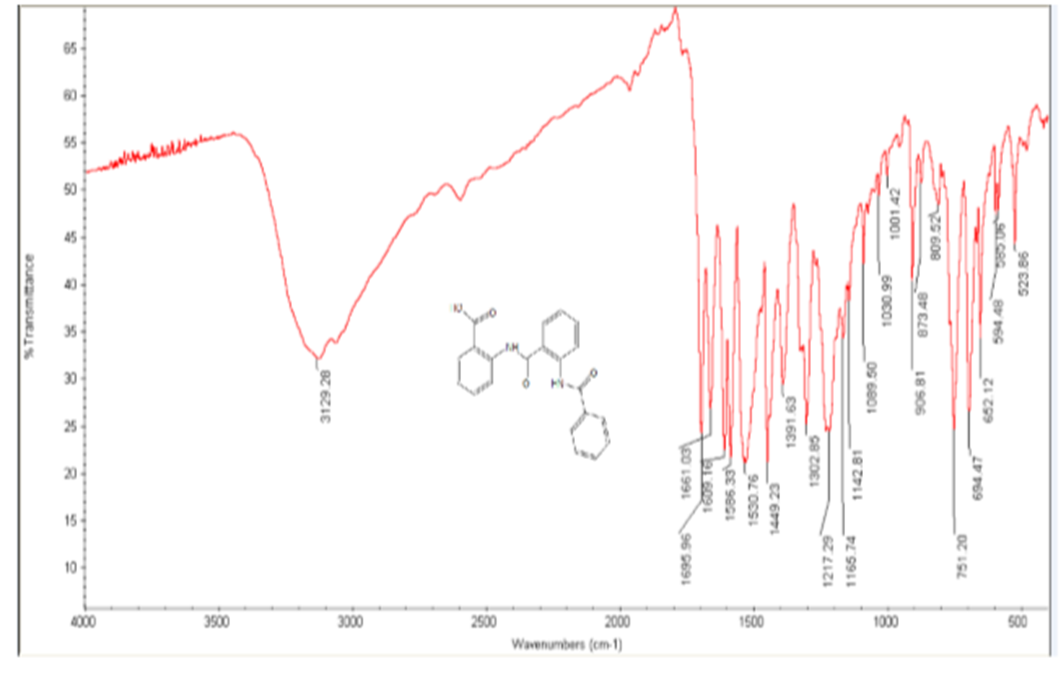
**Table S4** Calculated energy descriptors of **5 a-n** and **6 a-n**

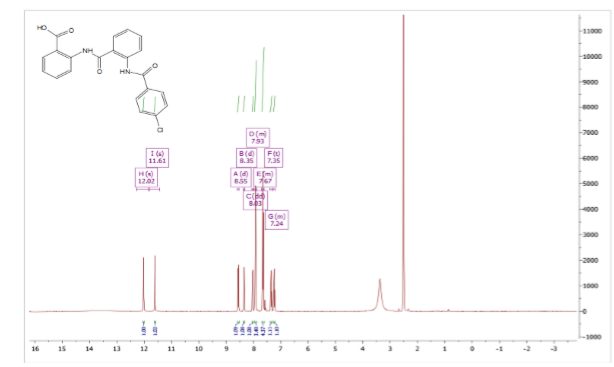
|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Compd. | IC50  (µM) | HOMO (eV) | LUMO  (eV) | ∆ E= (HOMO -LUMO) (eV) | I=  - EHOMO | A=  -ELUMO | χ=  (I+A)/2 | η=  (I-A)/2 | S=  1/2η | µ=  - (I+A)/2 | ω  = µ2/2η |
| 5a | 0.37 | -9.030 | -0.516 | -8.514 | 9.030 | 0.516 | 4.773 | 4.257 | 0.117 | -4.773 | 2.676 |
| 5b | 1.37 | -9.177 | -0.608 | -8.569 | 9.177 | 0.608 | 4.892 | 4.285 | 0.117 | -4.892 | 2.793 |
| 5c | 0.37 | -8.815 | -0.536 | -8.279 | 8.815 | 0.536 | 4.676 | 4.139 | 0.121 | -4.676 | 2.641 |
| 5d | 1.35 | -8.841 | -0.512 | -8.329 | 8.841 | 0.512 | 4.676 | 4.165 | 0.120 | -4.676 | 2.626 |
| 5e | 123.5 | -8.880 | -0.565 | -8.315 | 8.880 | 0.565 | 4.723 | 4.157 | 0.120 | -4.723 | 2.682 |
| 5f | 17.7 | -8.650 | -0.508 | -8.143 | 8.650 | 0.508 | 4.579 | 4.071 | 0.123 | -4.579 | 2.575 |
| 5g | 4.76 | -8.981 | -0.541 | -8.440 | 8.981 | 0.541 | 4.761 | 4.220 | 0.118 | -4.761 | 2.686 |
| 5h | 5.1 | -8.837 | -0.547 | -8.291 | 8.837 | 0.547 | 4.692 | 4.145 | 0.121 | -4.692 | 2.656 |
| 5i | 2.01 | -9.075 | -0.572 | -8.503 | 9.075 | 0.572 | 4.824 | 4.251 | 0.118 | -4.824 | 2.737 |
| 5j | 7.86 | -8.835 | -0.557 | -8.278 | 8.835 | 0.557 | 4.696 | 4.139 | 0.121 | -4.696 | 2.664 |
| 5k | 0.41 | -8.933 | -0.607 | -8.326 | 8.933 | 0.607 | 4.770 | 4.163 | 0.120 | -4.770 | 2.732 |
| 5l | 0.24 | -8.512 | -0.478 | -8.033 | 8.512 | 0.478 | 4.495 | 4.017 | 0.124 | -4.495 | 2.515 |
| 5m | 3.07 | -9.042 | -0.572 | -8.470 | 9.042 | 0.572 | 4.807 | 4.235 | 0.118 | -4.807 | 2.728 |
| 5n | 0.57 | -8.874 | -0.539 | -8.335 | 8.874 | 0.539 | 4.707 | 4.167 | 0.120 | -4.707 | 2.658 |
| 6a | 2.37 | -8.983 | -1.107 | -7.877 | 8.983 | 1.107 | 5.045 | 3.938 | 0.127 | -5.045 | 3.231 |
| 6b | 3.4 | -9.058 | -1.150 | -7.908 | 9.058 | 1.150 | 5.104 | 3.954 | 0.126 | -5.104 | 3.294 |
| 6c | 2.38 | -8.950 | -1.093 | -7.857 | 8.950 | 1.093 | 5.021 | 3.928 | 0.127 | -5.021 | 3.209 |
| 6d | 0.03 | -8.920 | -1.081 | -7.839 | 8.920 | 1.081 | 5.000 | 3.920 | 0.128 | -5.000 | 3.190 |
| 6e | 1.24 | -8.968 | -1.156 | -7.812 | 8.968 | 1.156 | 5.062 | 3.906 | 0.128 | -5.062 | 3.280 |
| 6f | 0.66 | -8.807 | -1.087 | -7.720 | 8.807 | 1.087 | 4.947 | 3.860 | 0.130 | -4.947 | 3.170 |
| 6g | 0.72 | -9.093 | -1.111 | -7.982 | 9.093 | 1.111 | 5.102 | 3.991 | 0.125 | -5.102 | 3.261 |
| 6h | 0.36 | -8.973 | -1.121 | -7.852 | 8.973 | 1.121 | 5.047 | 3.926 | 0.127 | -5.047 | 3.244 |
| 6i | 1.21 | -9.086 | -1.187 | -7.899 | 9.086 | 1.187 | 5.137 | 3.950 | 0.127 | -5.137 | 3.340 |
| 6j | 0.43 | -8.972 | -1.122 | -7.851 | 8.972 | 1.122 | 5.047 | 3.925 | 0.127 | -5.047 | 3.245 |
| 6k | 0.44 | -8.980 | -1.165 | -7.815 | 8.980 | 1.165 | 5.073 | 3.907 | 0.128 | -5.073 | 3.292 |
| 6l | 0.08 | -8.679 | -1.153 | -7.526 | 8.679 | 1.153 | 4.916 | 3.763 | 0.133 | -4.916 | 3.212 |
| 6m | 0.42 | -9.148 | -1.172 | -7.977 | 9.148 | 1.172 | 5.160 | 3.988 | 0.125 | -5.160 | 3.338 |
| 6n | 3.25 | -8.988 | -1.134 | -7.855 | 8.988 | 1.134 | 5.061 | 3.927 | 0.127 | -5.061 | 3.261 |

**Figure S1 1H NMR spectrum of 5a**

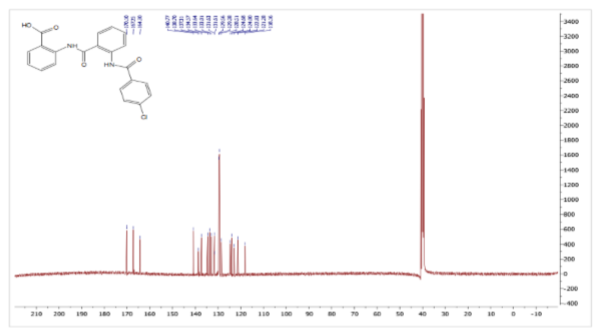


**Figure S2 13CNMR spectrum of 5a**

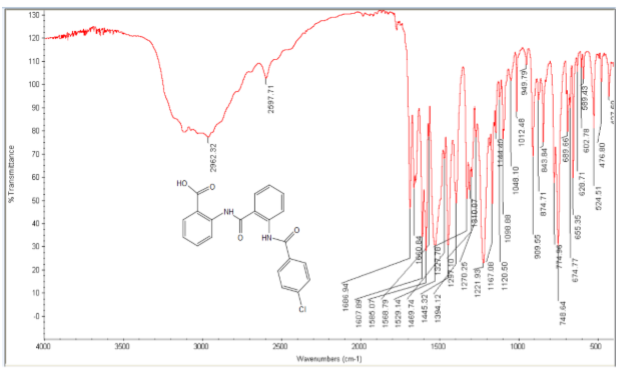
**Figure S3 FT-IR spectrum 5a**

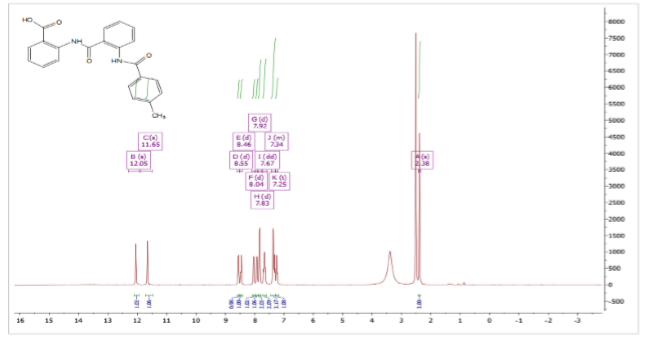
**Figure S4 1H NMR spectrum of 5b**

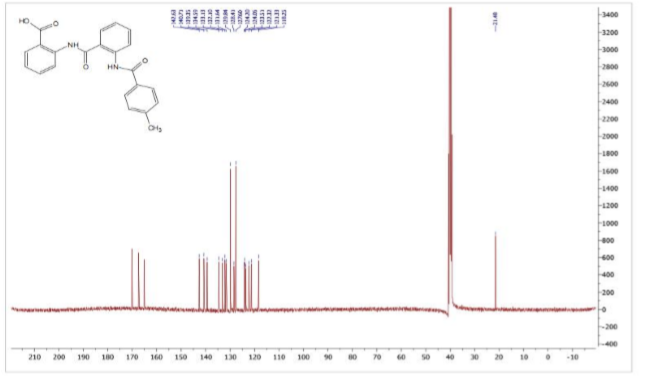
**Figure S5 13CNMR spectrum of 5b**



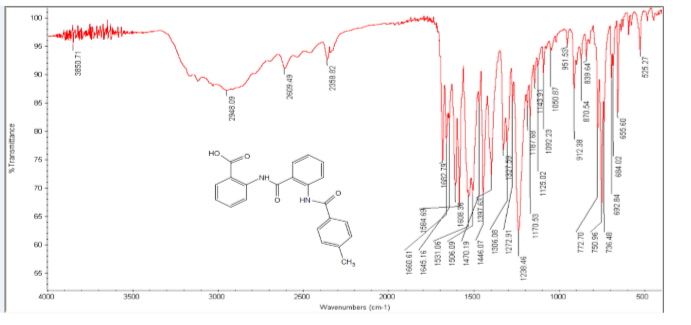
**Figure S6 FT-IR spectrum of 5b**



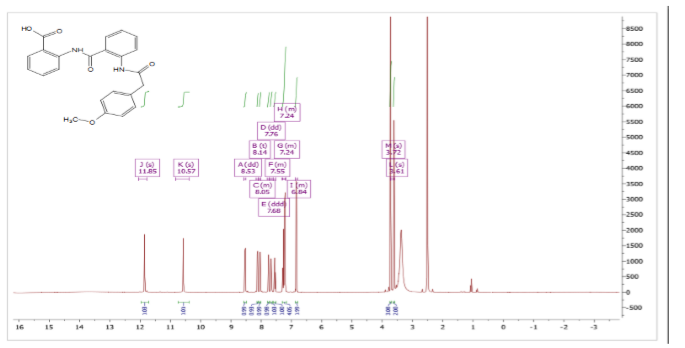
**Figure S7 1H NMR spectrum of 5c**

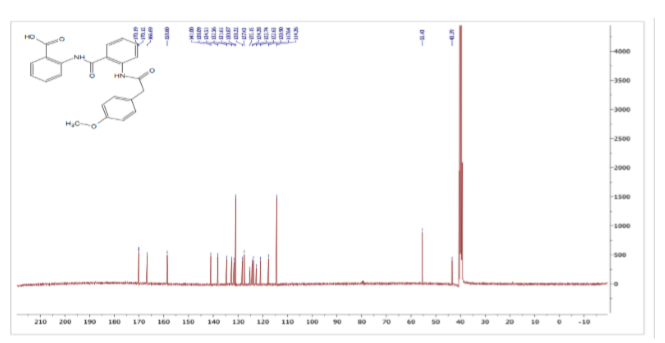
**Figure S8 13NMR spectrum of 5c**

**Figure S9 Ft-IR spectrum 5c**

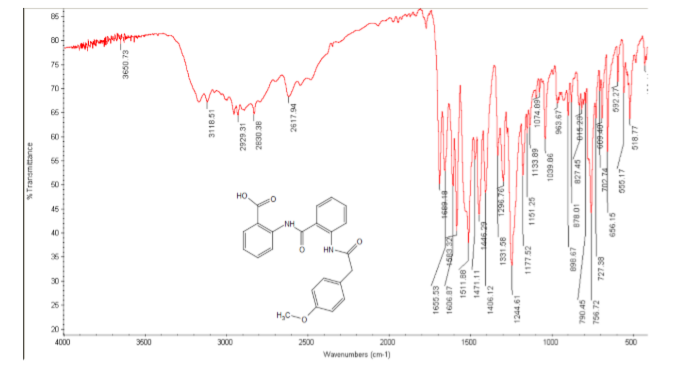


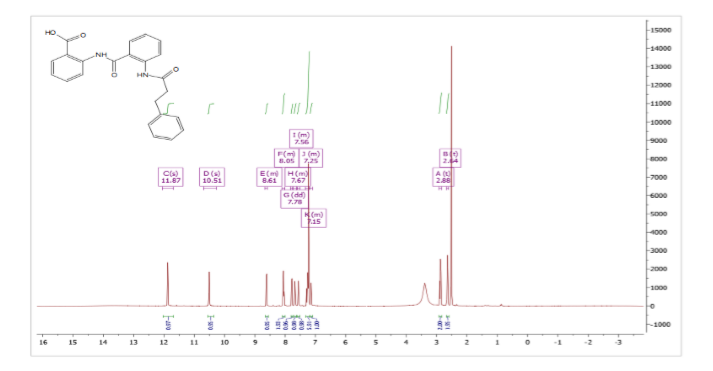
**Figure S101H NMR spectrum of 5d**



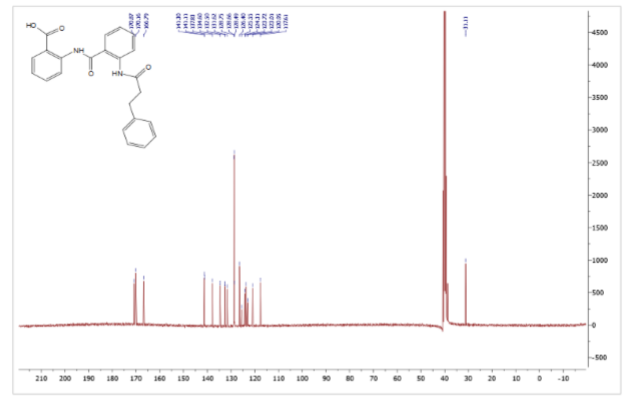
**Figure S11 13CNMR spectrum of 5d**

**Figure S12 FT-IR spectrum of 5d**

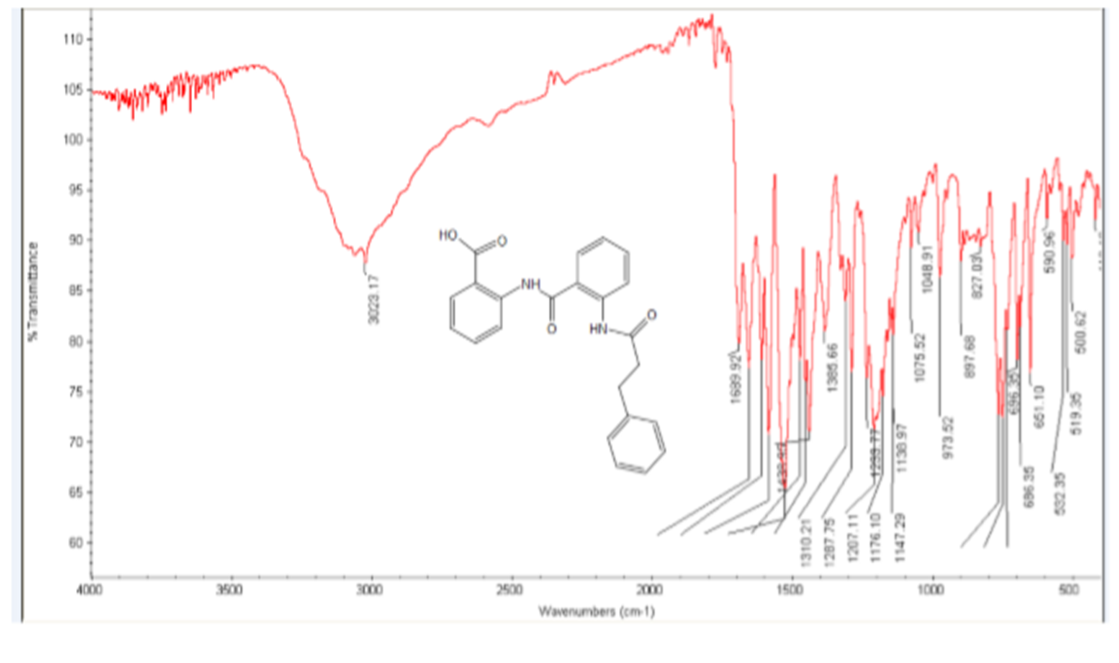


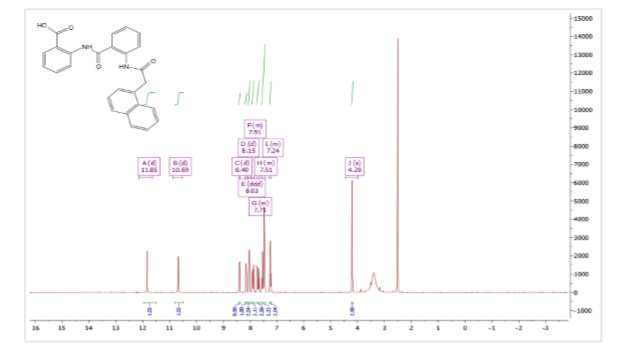
**Figure S13 1H NMR spectrum of 5e**

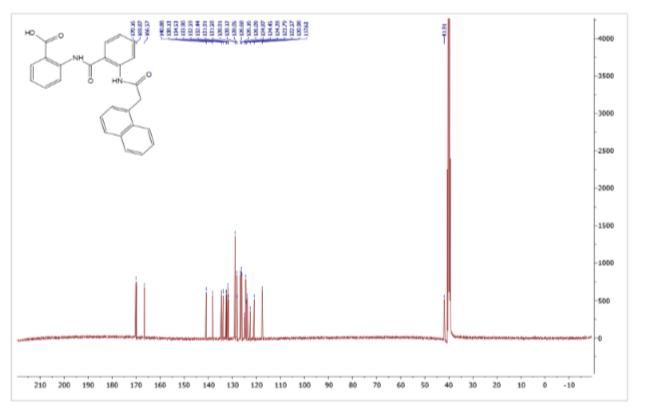
**Figure S14 13CNMR spectrum of 5e**



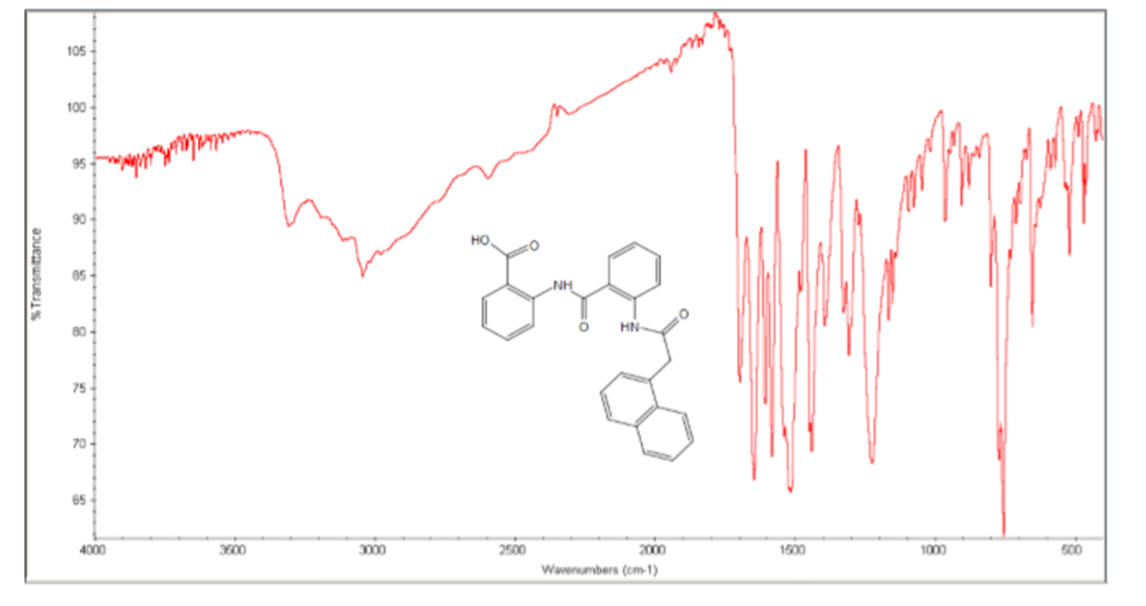
**Figure S15 FT-IR spectrum of 5e**



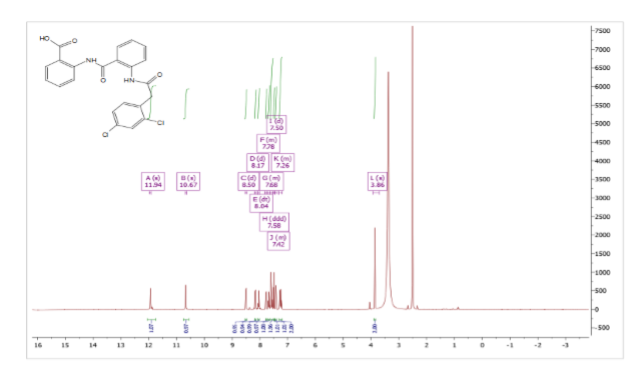
**Figure S16 1H NMR spectrum of 5f**

**Figure S1713CNMR spectrum of 5f**

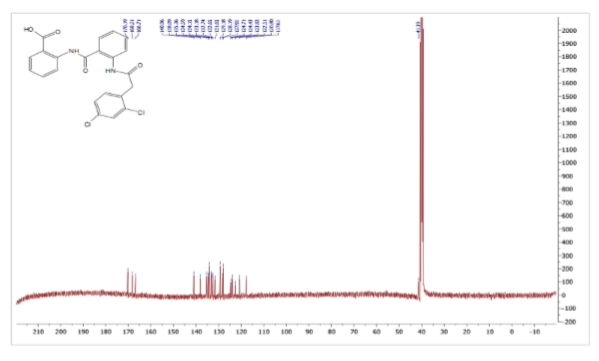
**Figure S18 FT-IR spectrum of 5f**



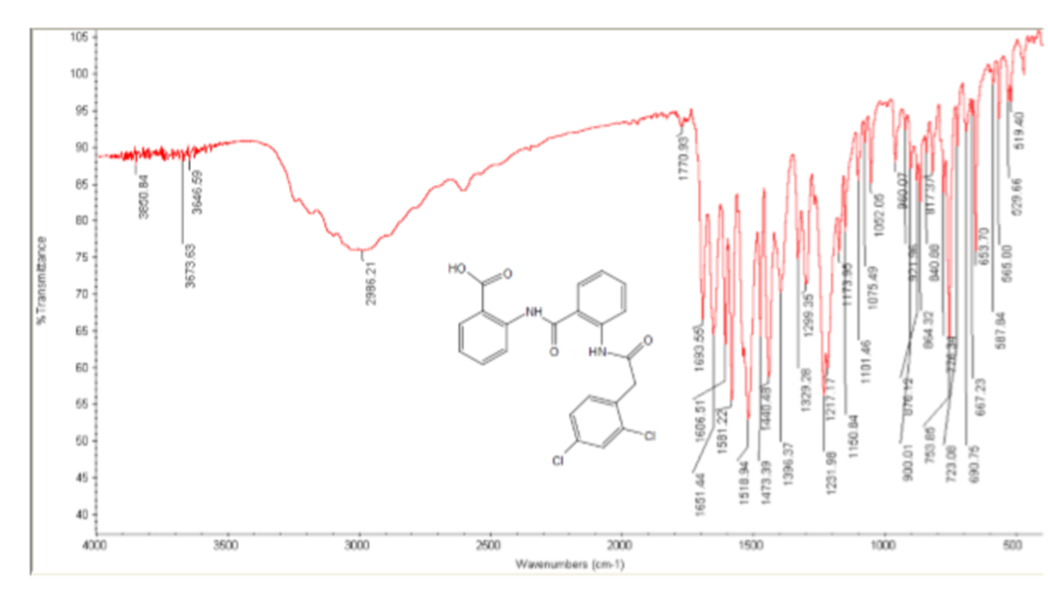
**Figure S19 1H NMR spectrum of5g**



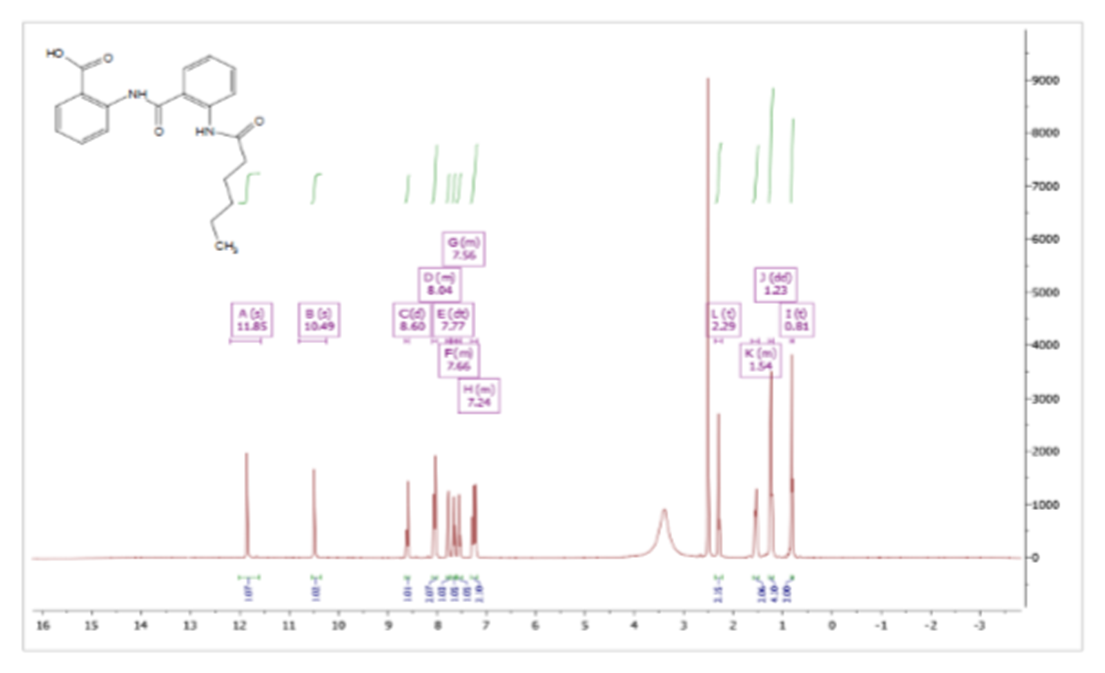
**Figure S20 13CNMR spectrum of5g**

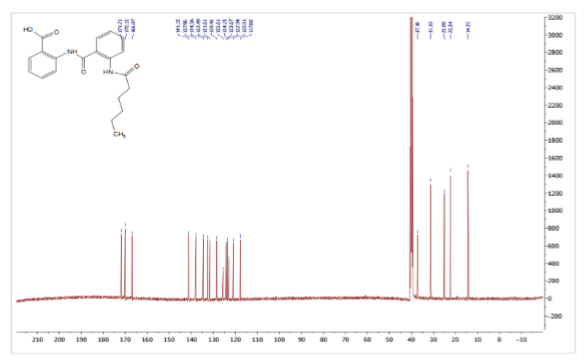
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**Figure S21 FT-IR spectrum of5g**

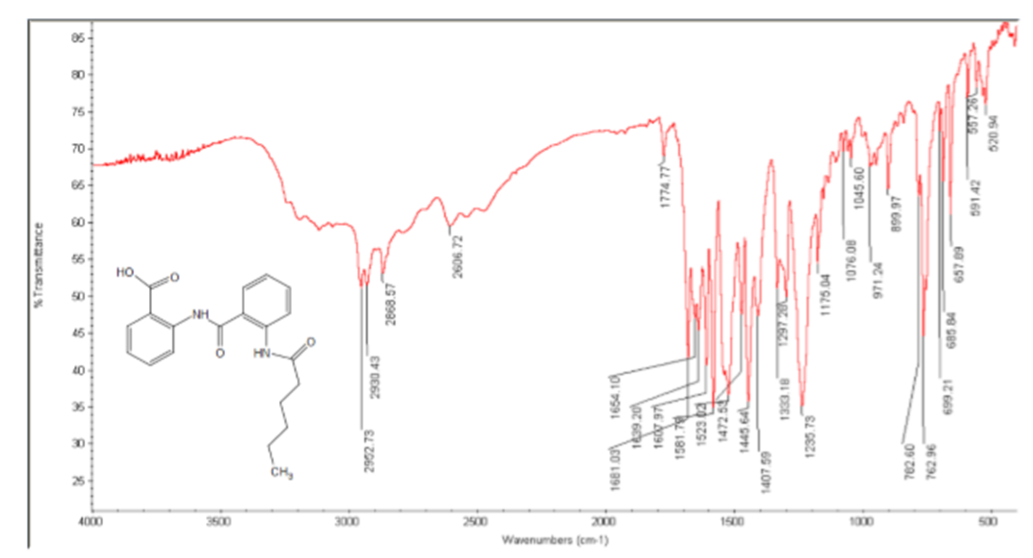


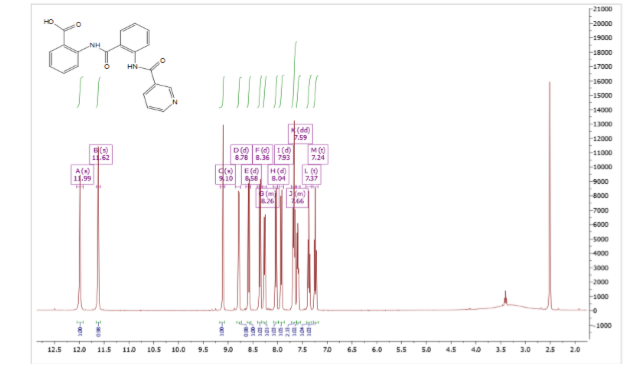
**Figure S22 1H NMR spectrum of5h**



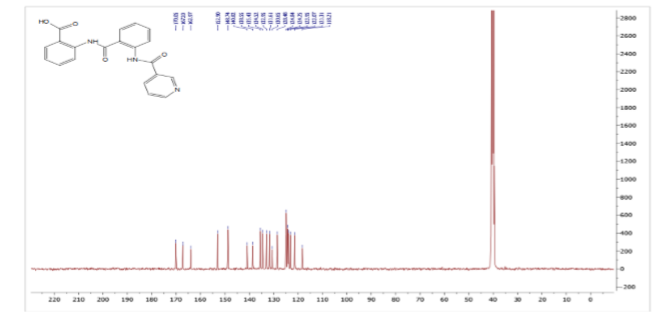
**Figure S23 13CNMR spectrum of5h**

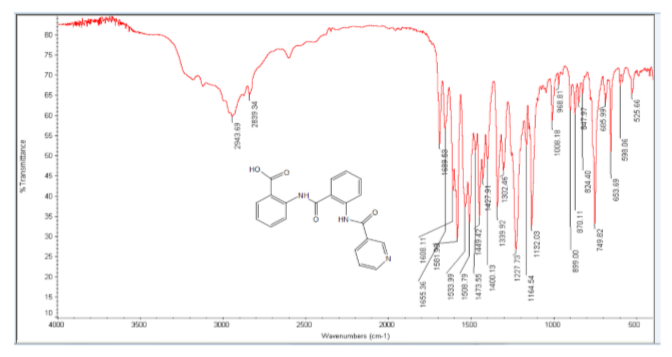
**Figure S24 FT-IR spectrum of5h**

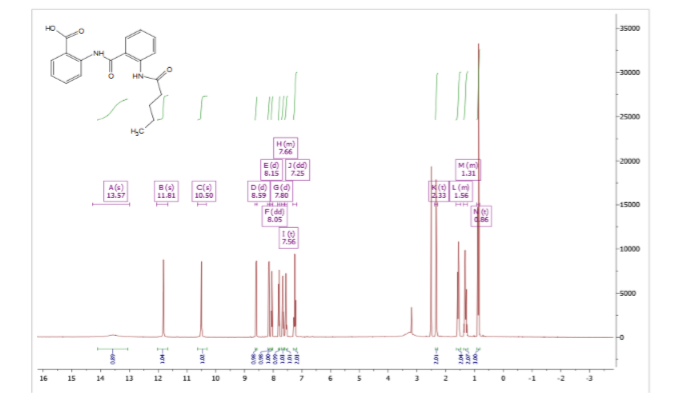


**Figure S25 1H NMR spectrum of5i**

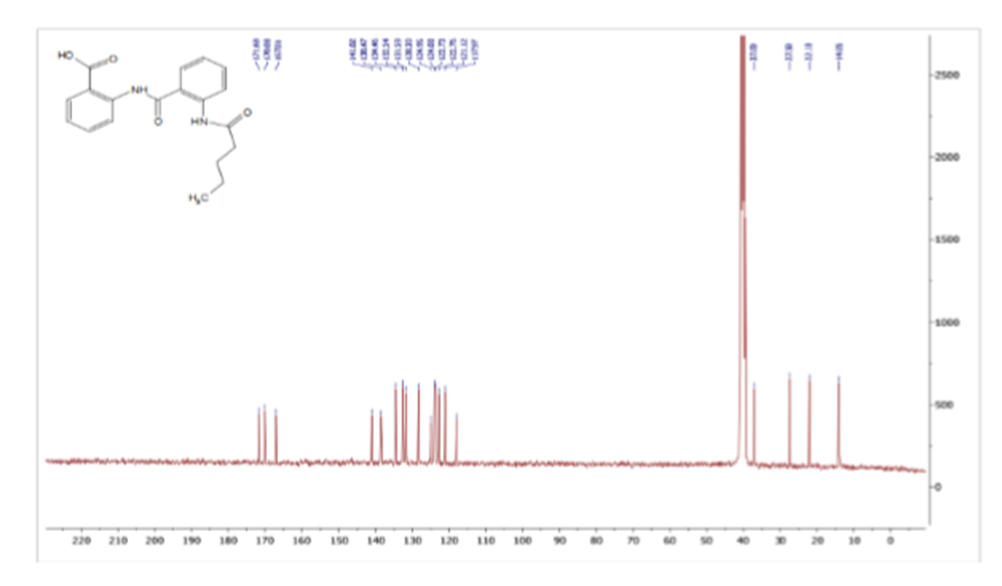
**Figure S26 13CNMR spectrum of5i**



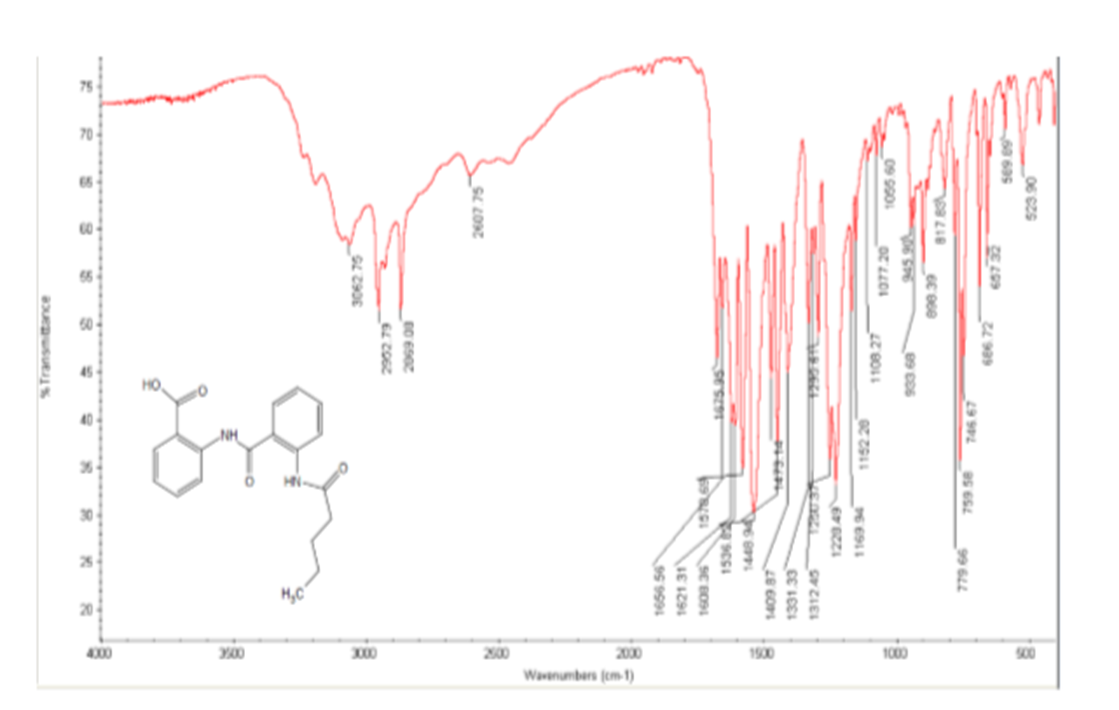
**Figure S27 FT-IR spectrum of5i**

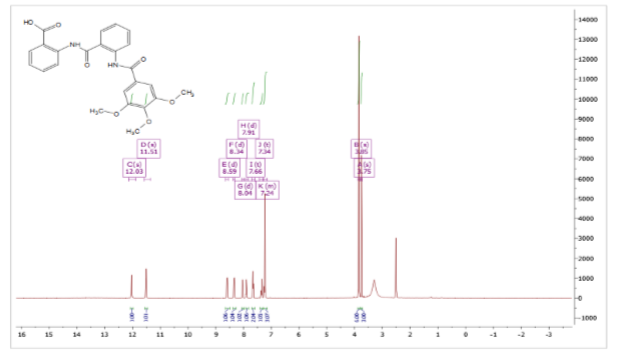
**Figure S28 1H NMR spectrum of5j**

**Figure S29 13CNMR spectrum of5j**

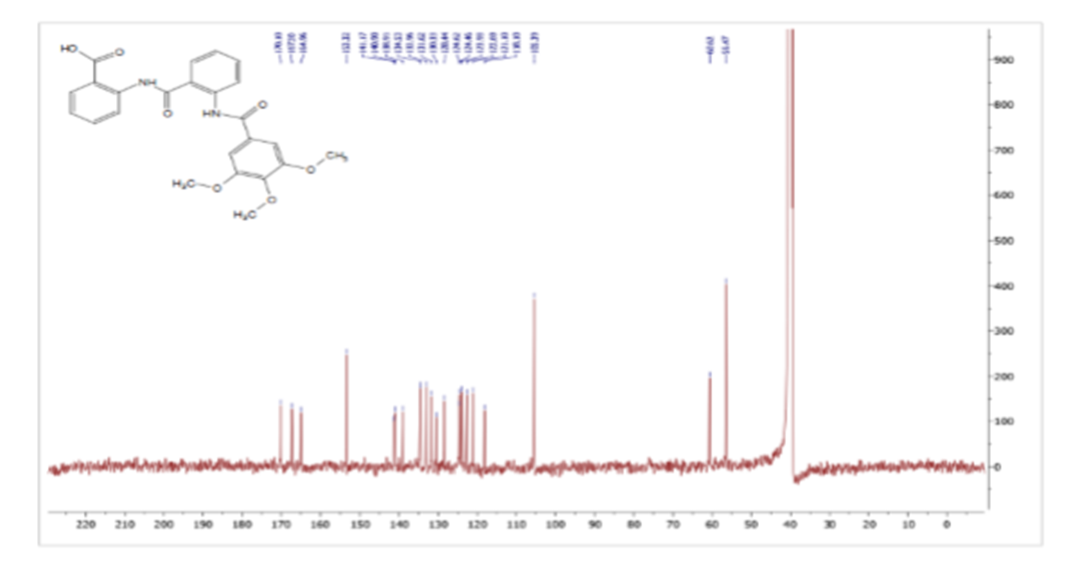


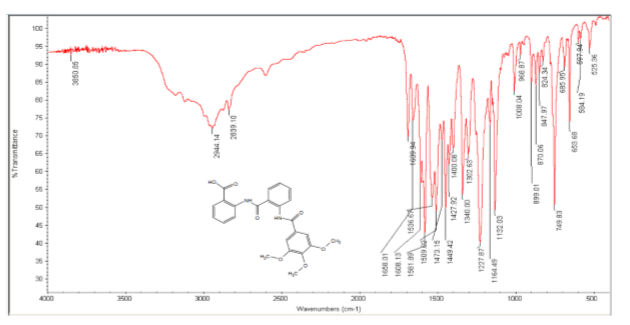
**Figure S30 FT-IR spectrum of5j**



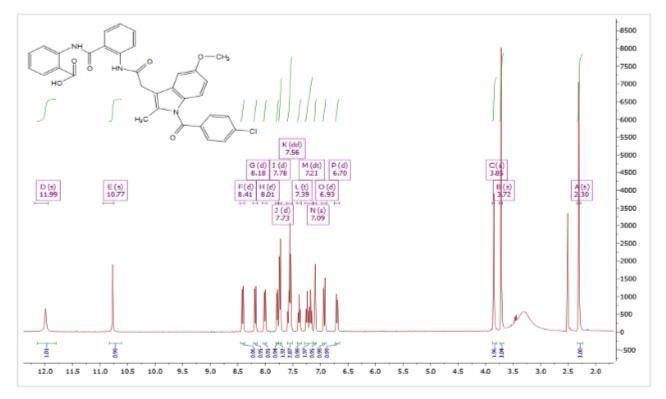
**Figure S31 1H NMR spectrum of 5k**

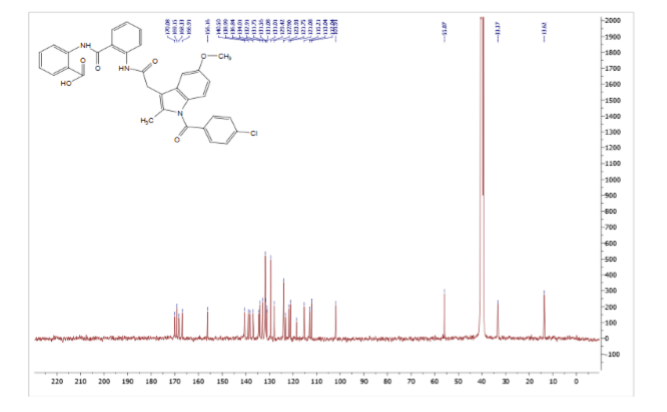
**Figure S32 13CNMR spectrum of5k**



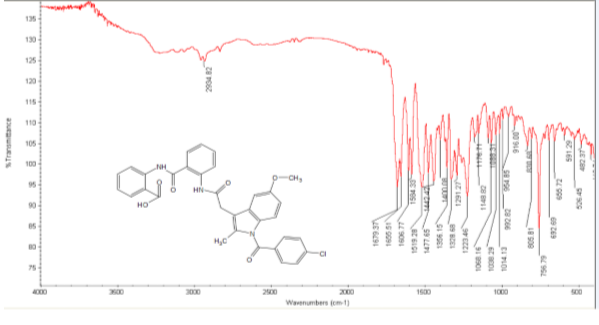
**Figure S33 FT-IR spectrum of 5k**

**Figure S341H NMR spectrum of 5l**

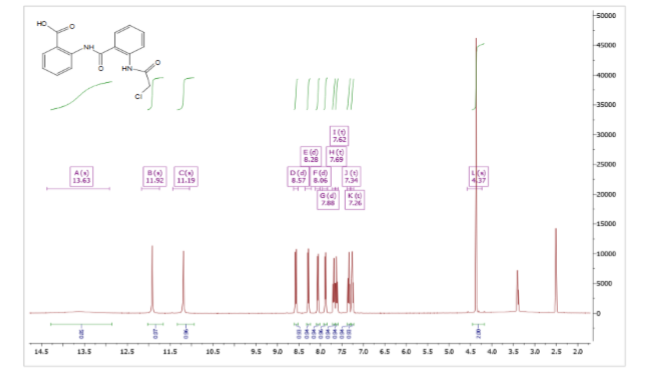


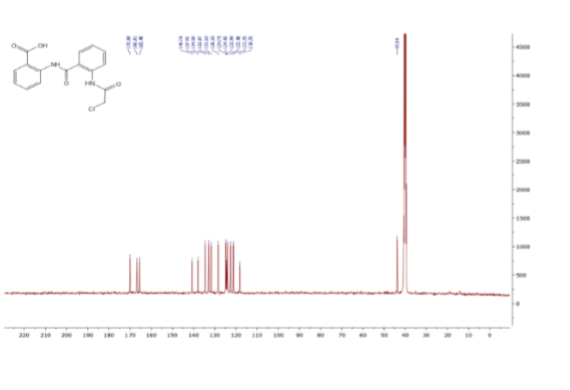
**Figure S3513CNMR spectrum of 5l**

**Figure S36FT-IR spectrum of 5l**

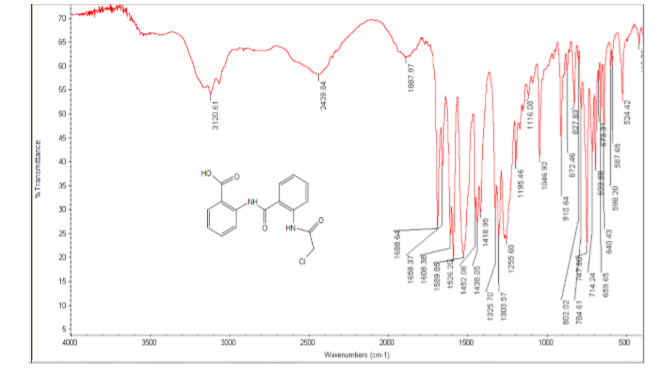


**Figure S371H NMR spectrum of 5m**

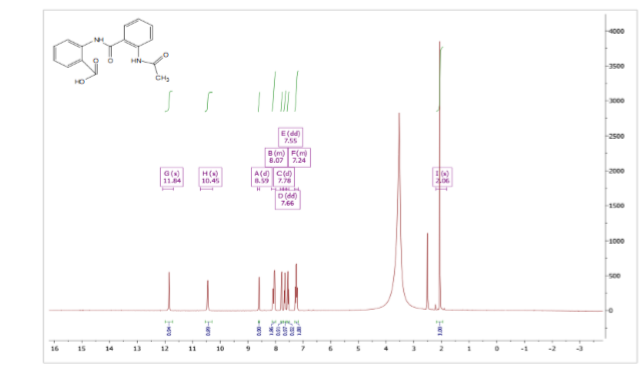


**Figure S3813CNMR spectrum of 5m**

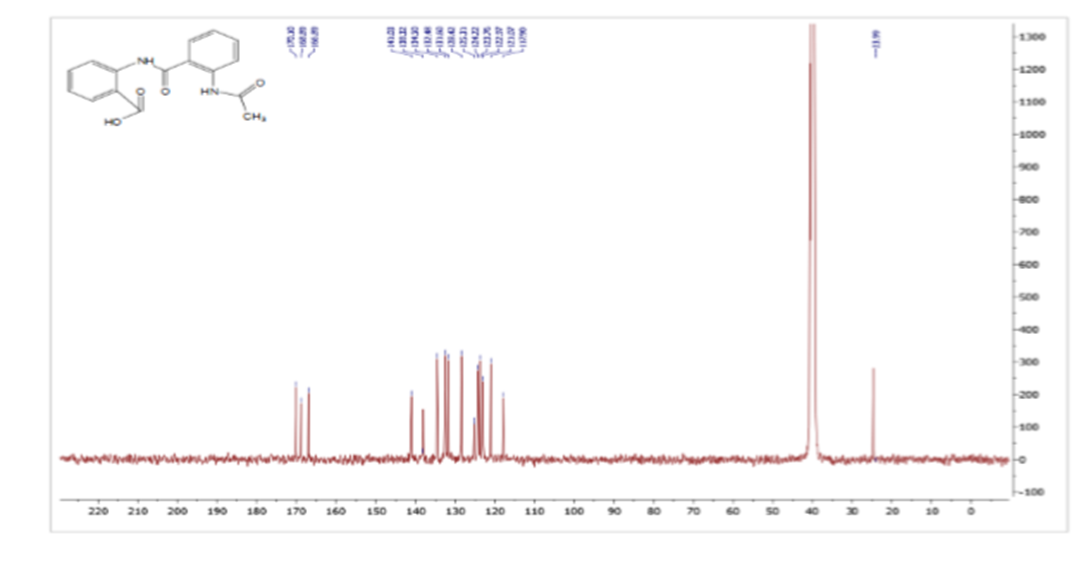
**Figure S39FT-IR spectrum of 5m**



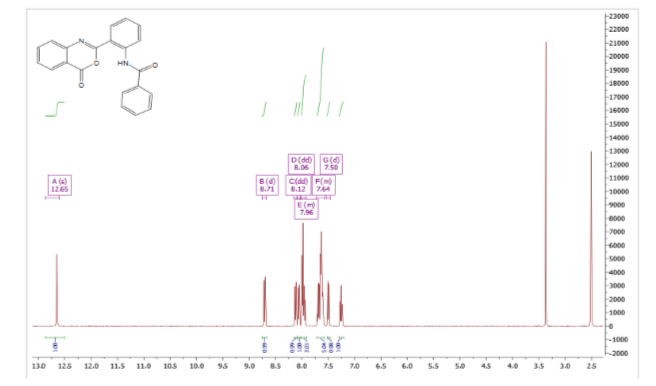
**Figure S401H NMR spectrum of 5n**



**Figure S41 13CNMR spectrum of 5n**



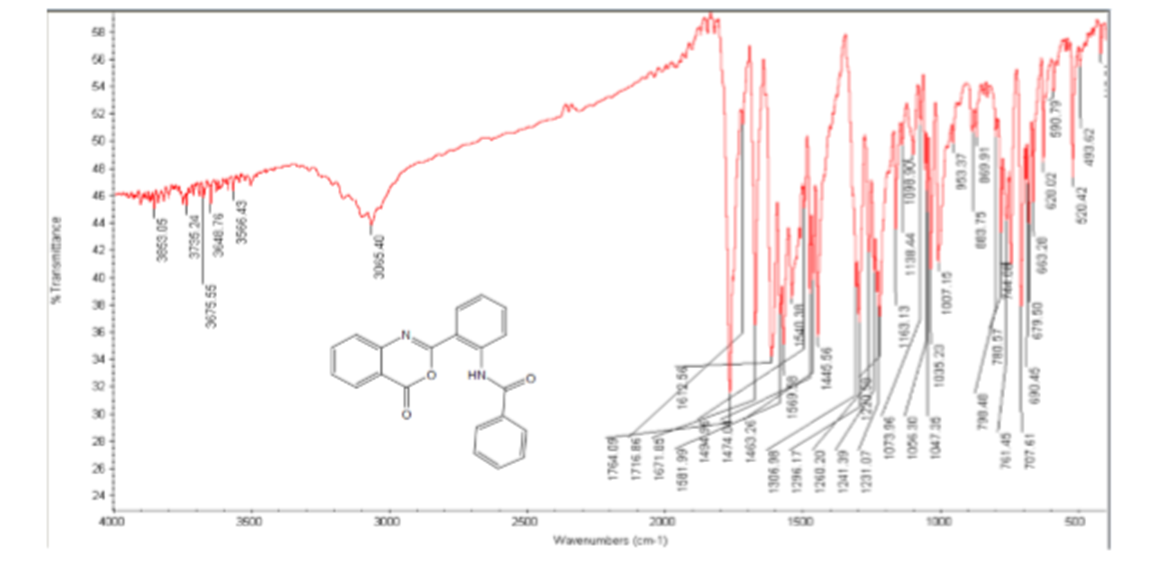
**Figure S421H NMR spectrum of 6a**



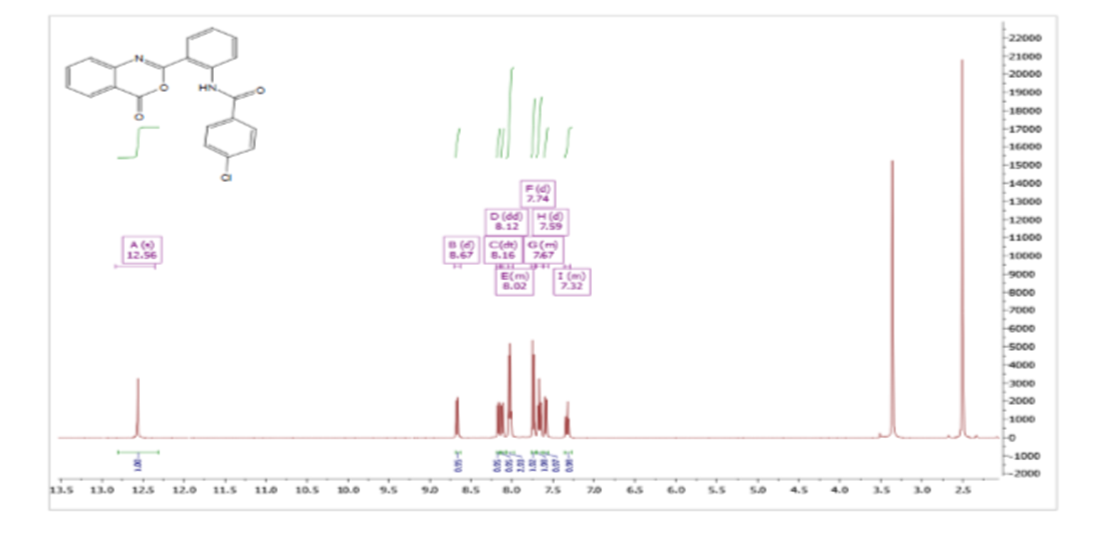
**Figure S4313CNMR spectrum of 6a**

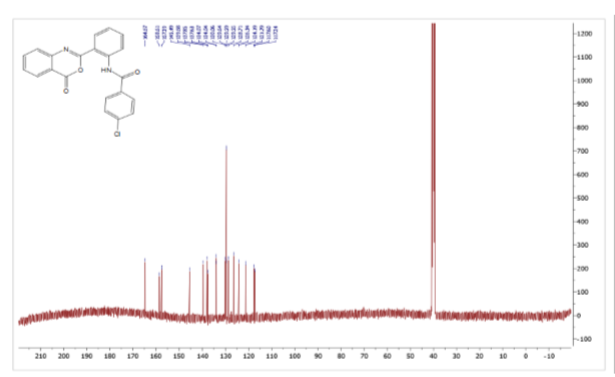


**Figure S44FT-IR spectrum of 6a**

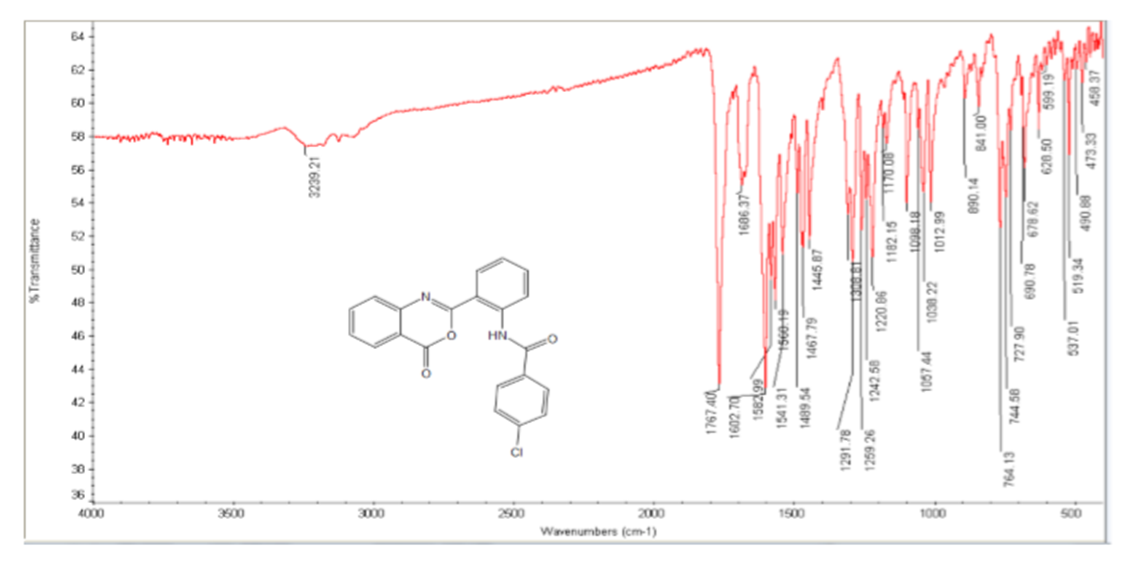


**Figure S451H NMR spectrum of 6b**

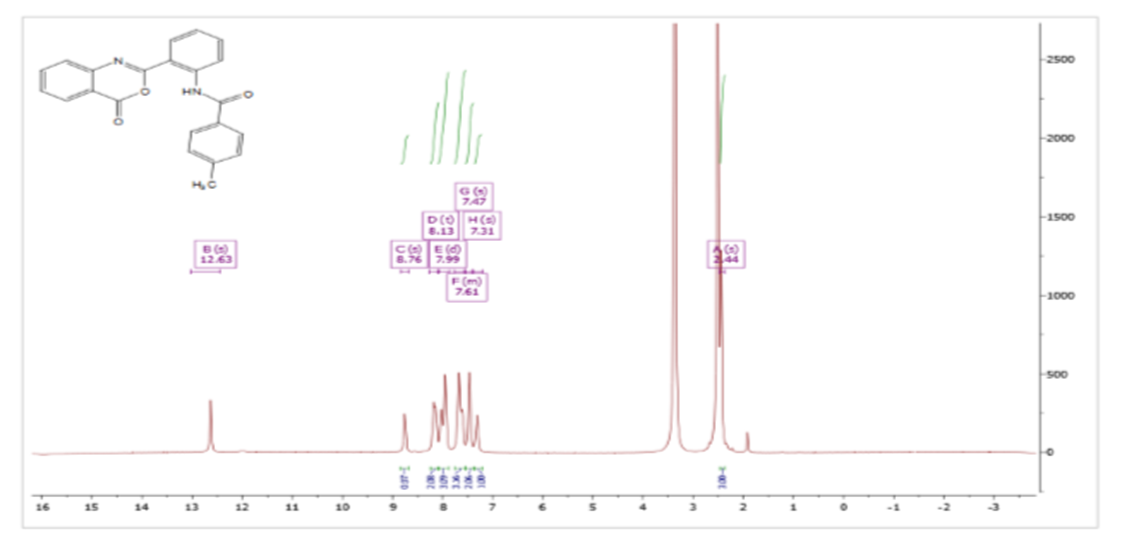


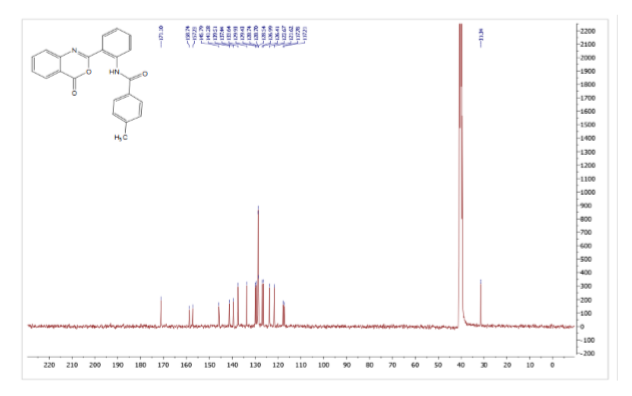
**Figure S46 13CNMR spectrum of 6b**

**Figure S47 FT-IR spectrum of 6b**

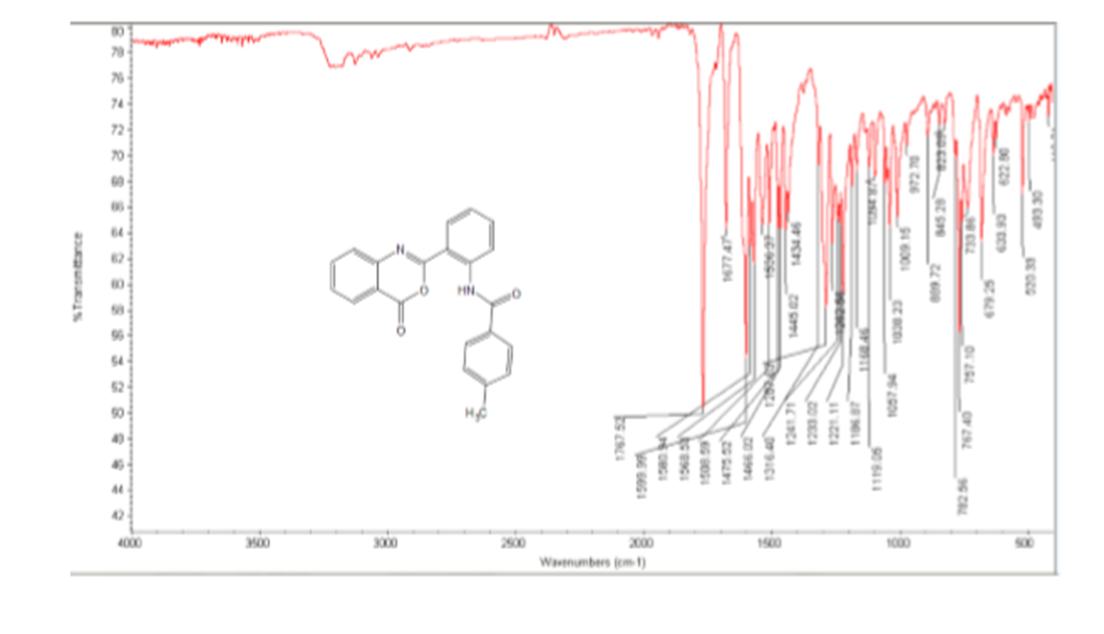


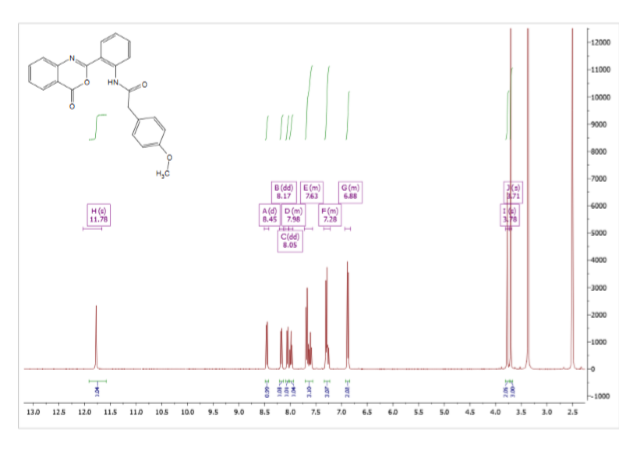
**Figure S48 1H NMR spectrum of 6c**



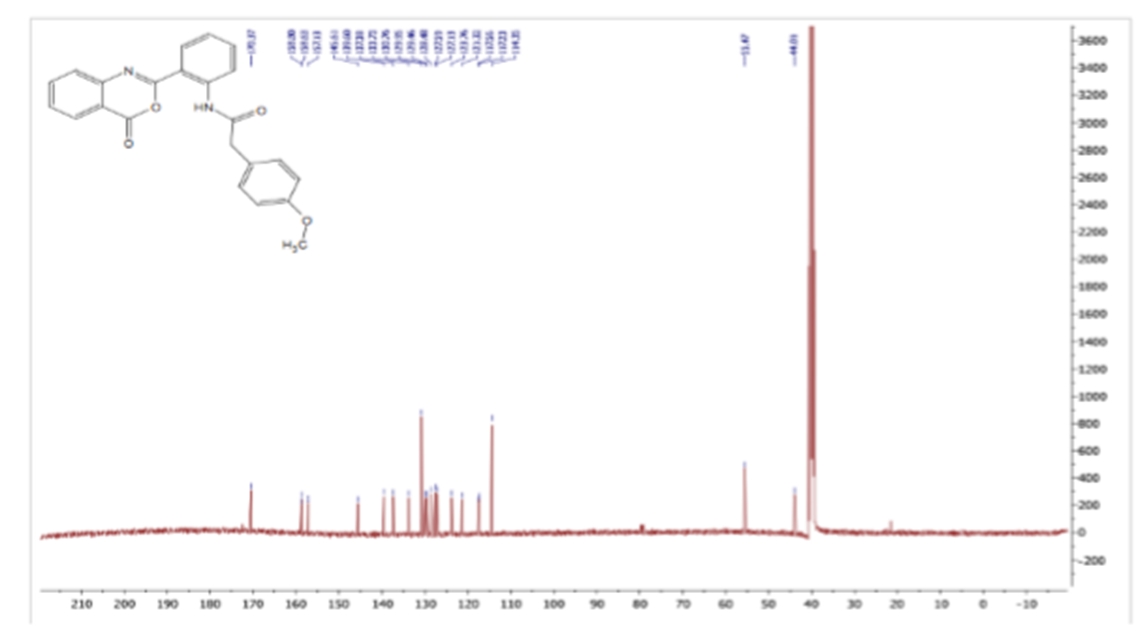
**Figure S49 13CNMR spectrum of 6c**

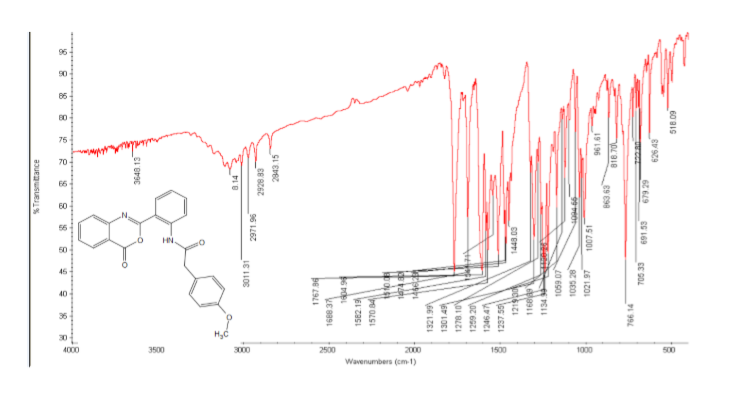
**Figure S50 FT-IR spectrum of 6c**



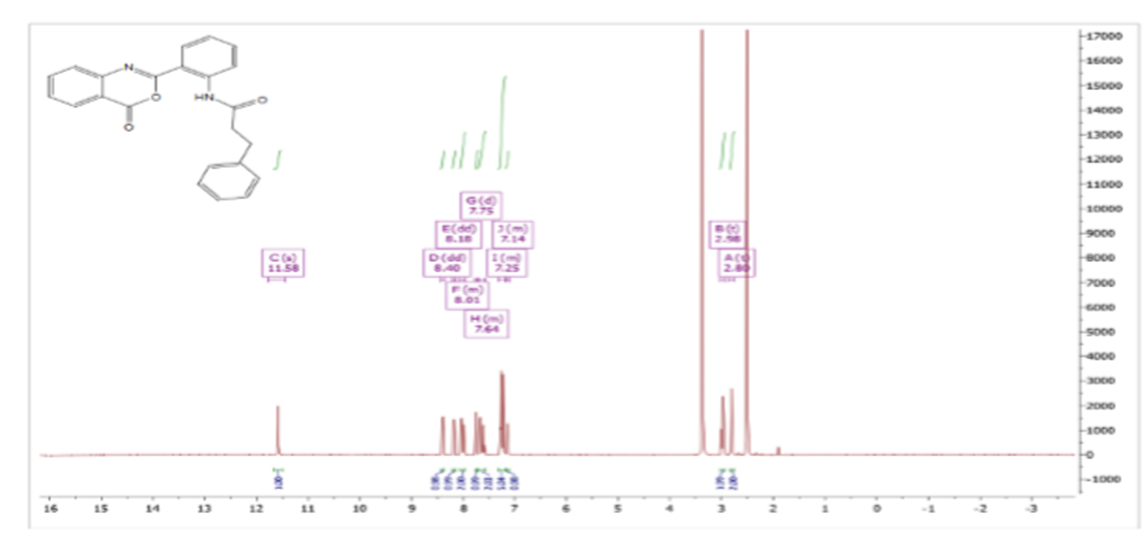
**Figure S51 1H NMR spectrum of 6d**

**Figure S52 13CNMR spectrum of 6d**

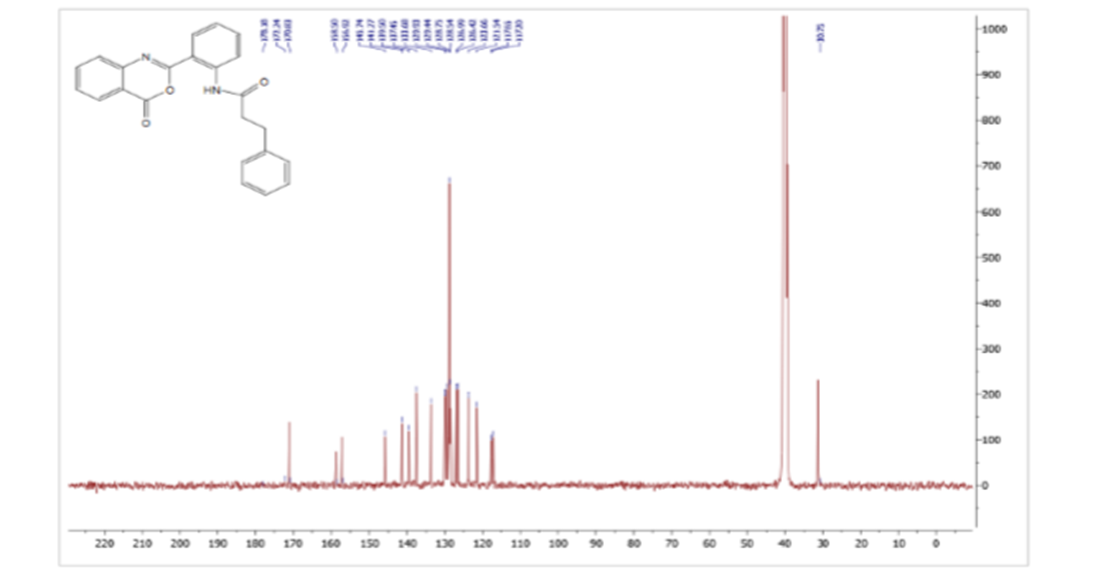


**Figure S53 FT-IR spectrum of 6d**

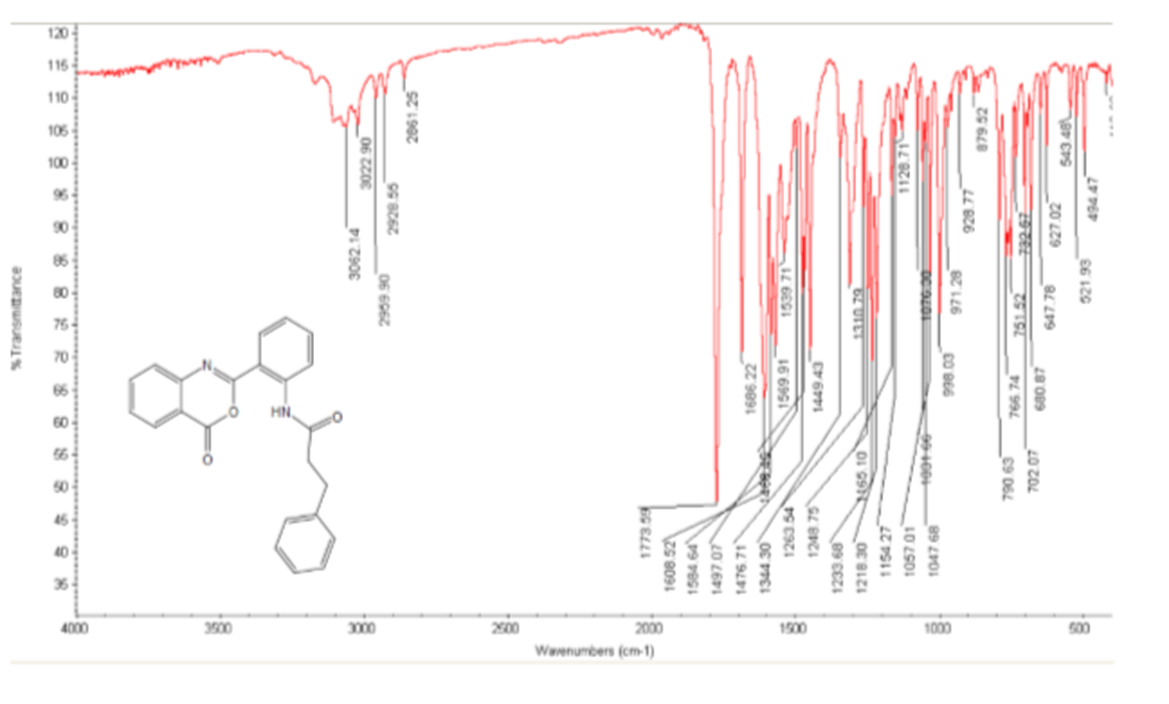
**Figure S54 1H NMR spectrum of 6e**



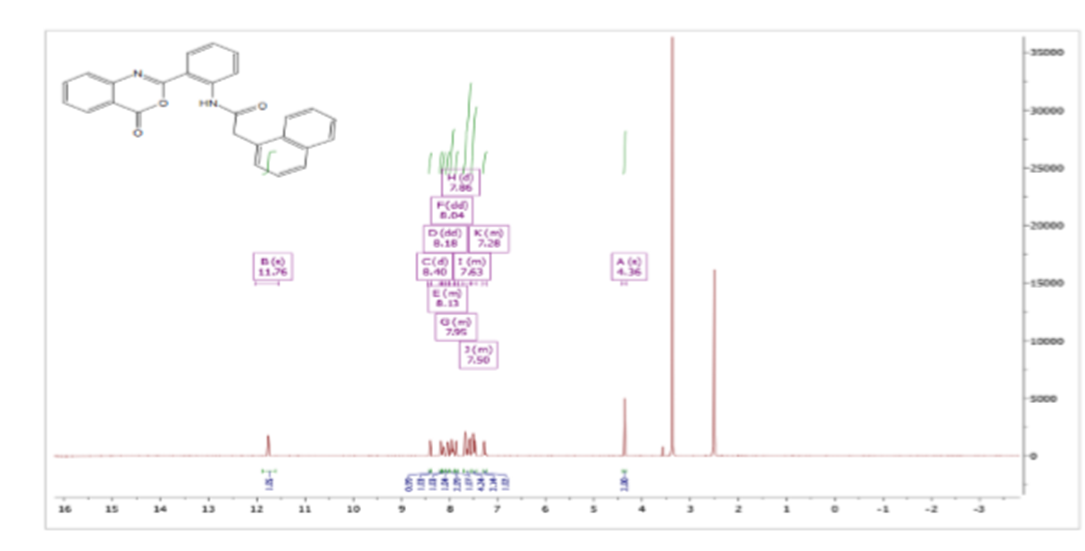
**Figure S55 13CNMR spectrum of 6e**



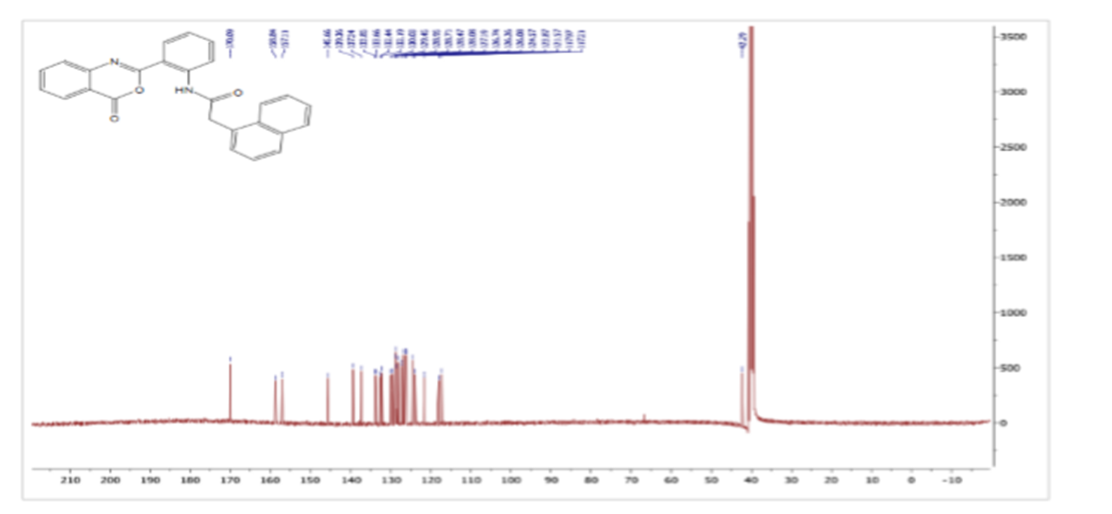
**Figure S56 FT-IR spectrum of 6e**

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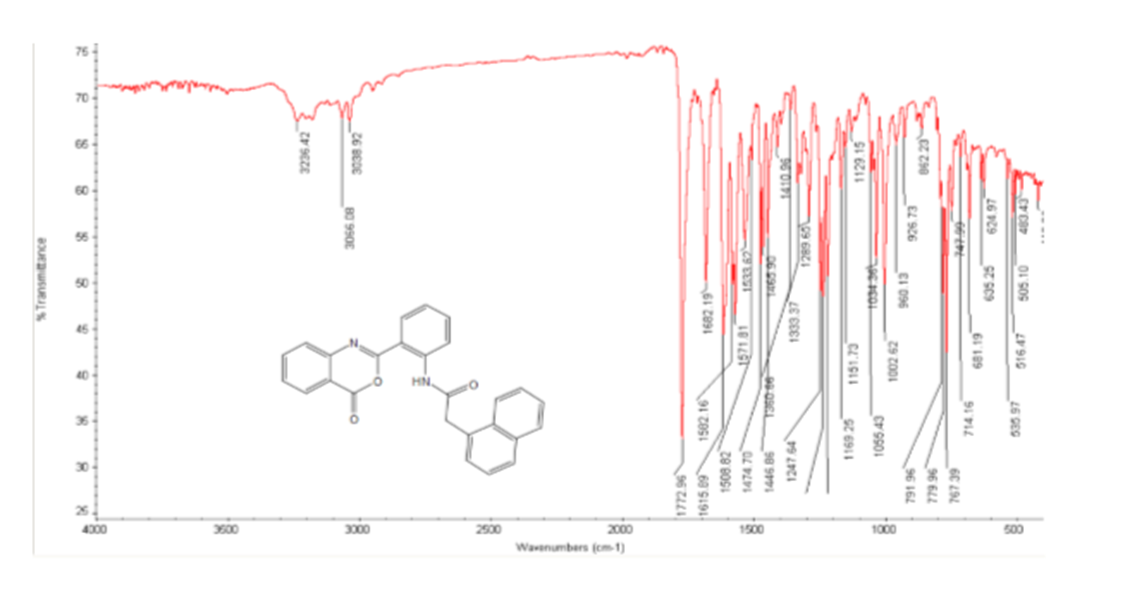
**Figure S57 1H NMR spectrum of 6f**



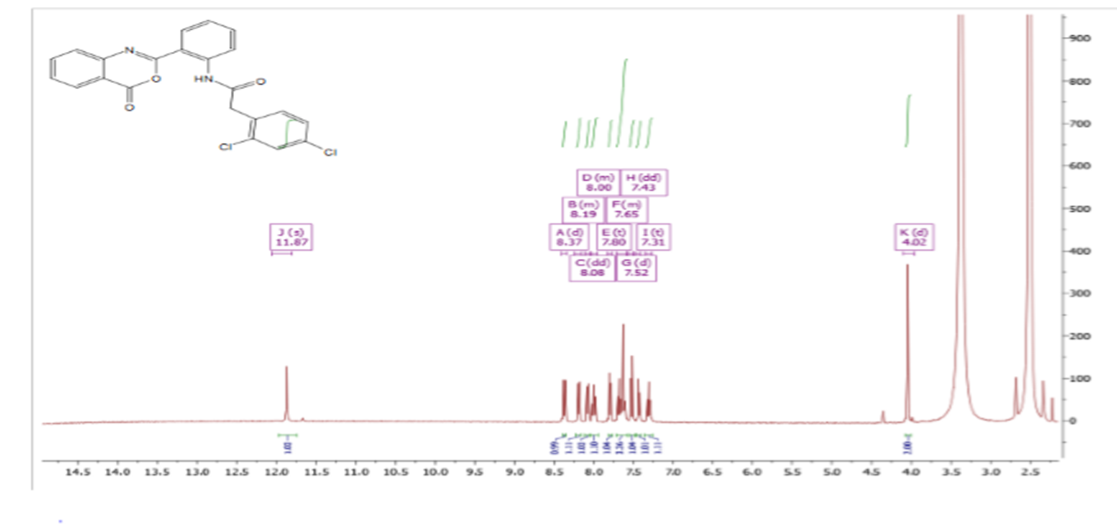
**Figure S58 13CNMR spectrum of 6f**



**Figure S59 FT-IR spectrum of 6f**

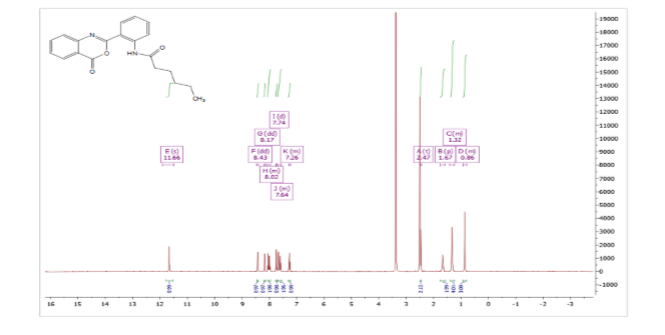


**Figure S60 1H NMR spectrum of 6g**

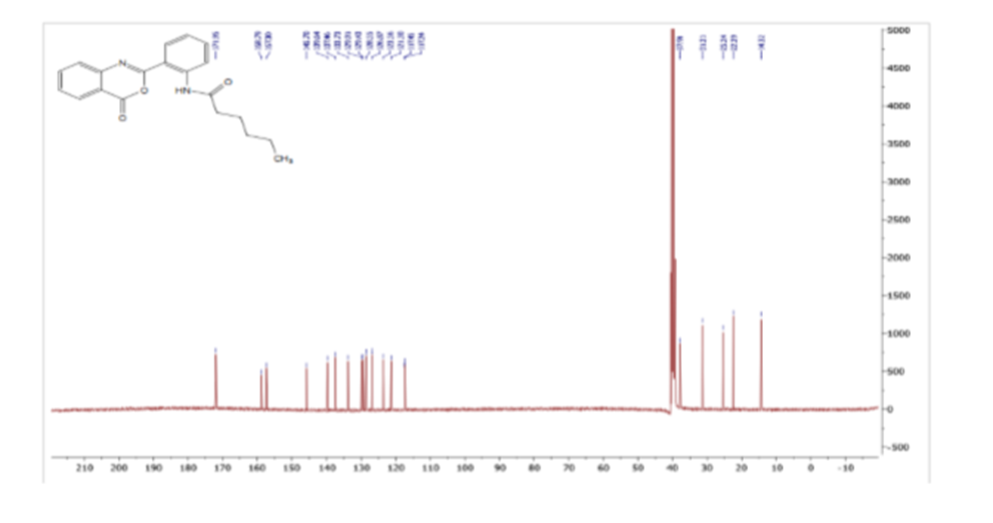


**Figure S61 13CNMR spectrum of 6g**

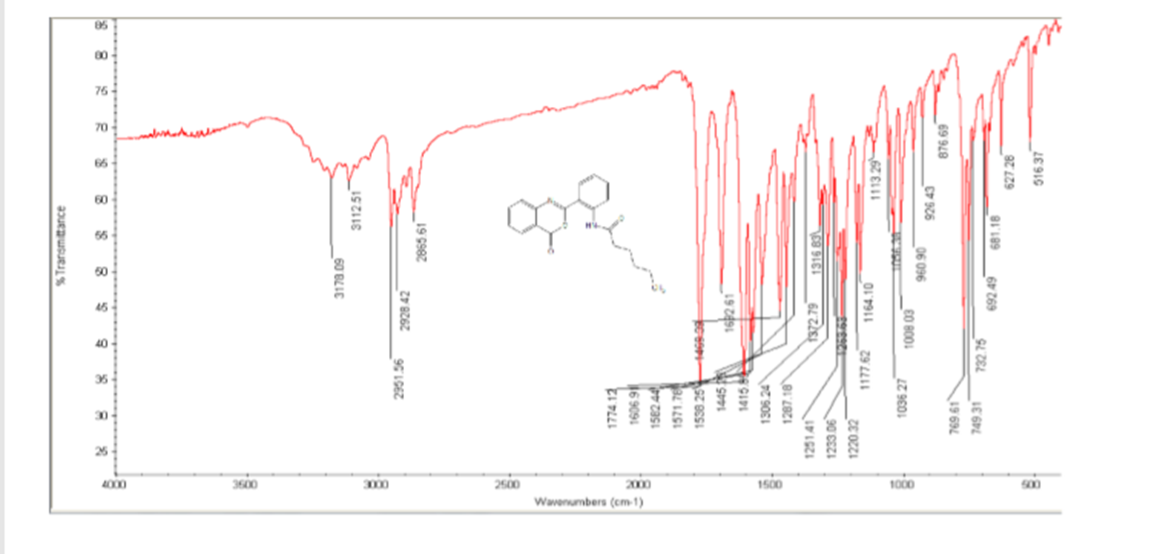


**Figure S62 1H NMR spectrum of 6h**

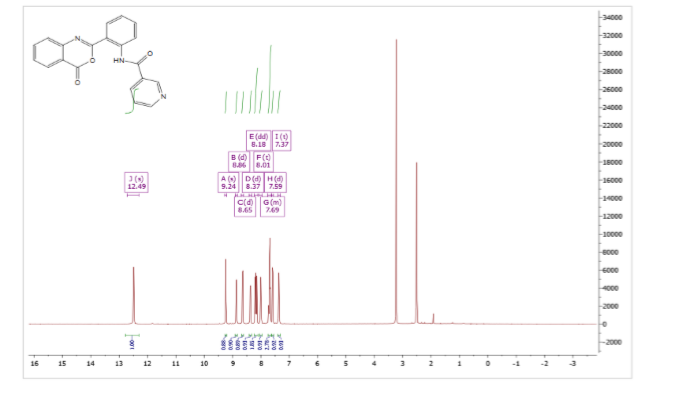
**Figure S63 13CNMR spectrum of 6h**



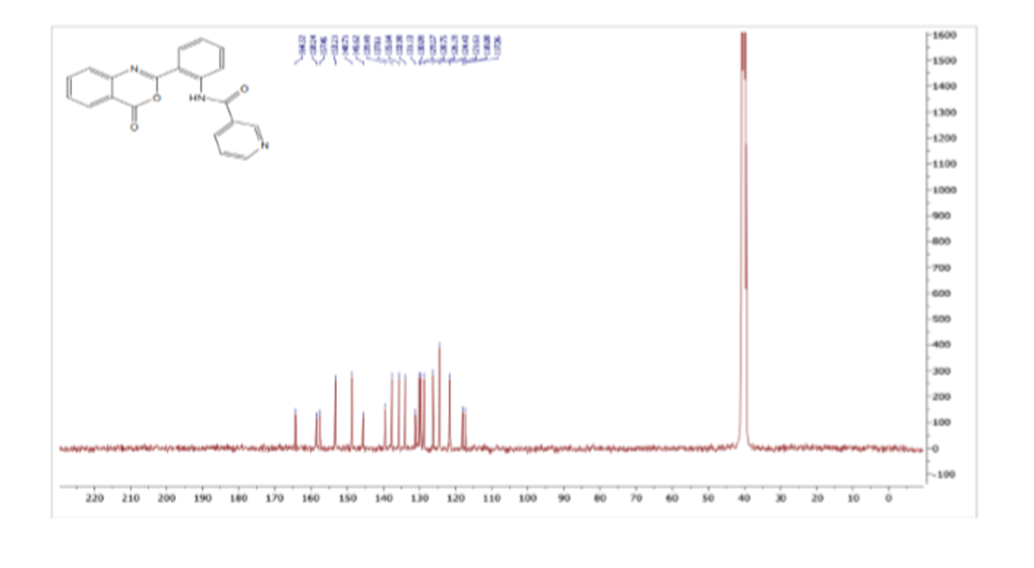
**Figure S64 FT-IR spectrum of 6h**

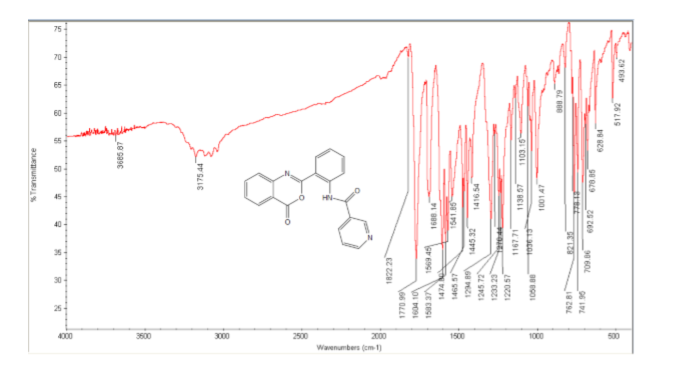


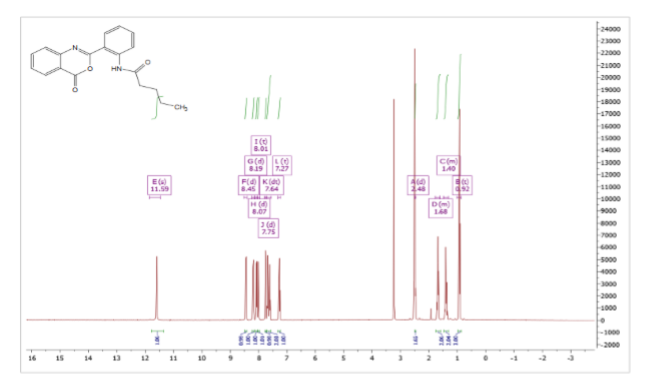
**Figure S651H NMR spectrum of 6i**

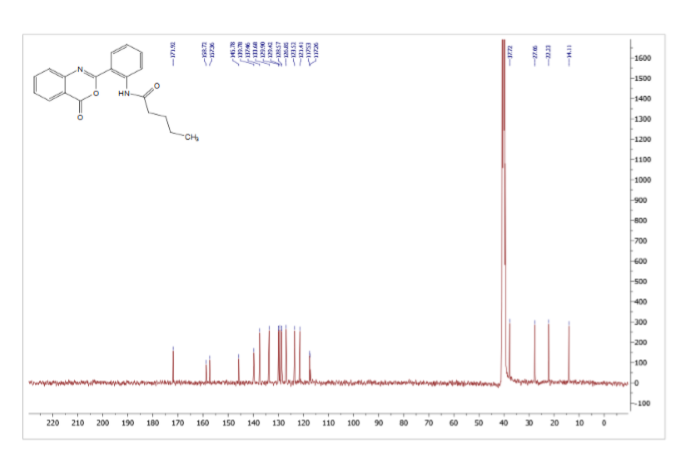


**Figure S6613CNMR spectrum of 6i**

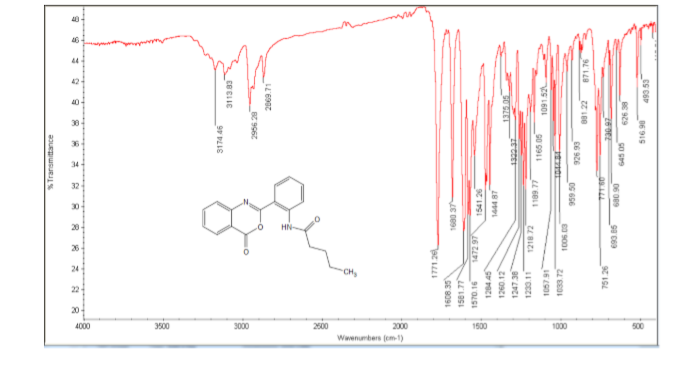


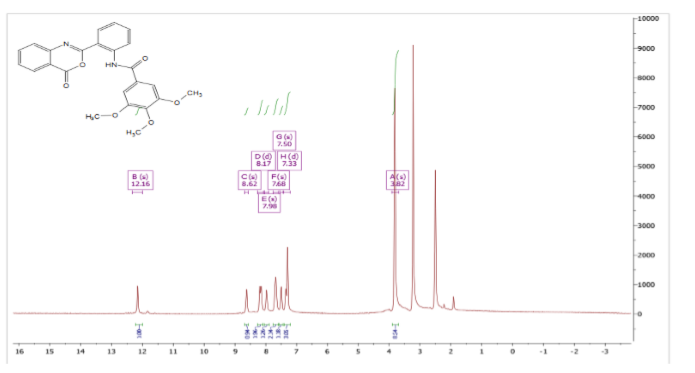
**Figure S67 FT-IR spectrum of 6i**

**Figure S681H NMR spectrum of 6j**

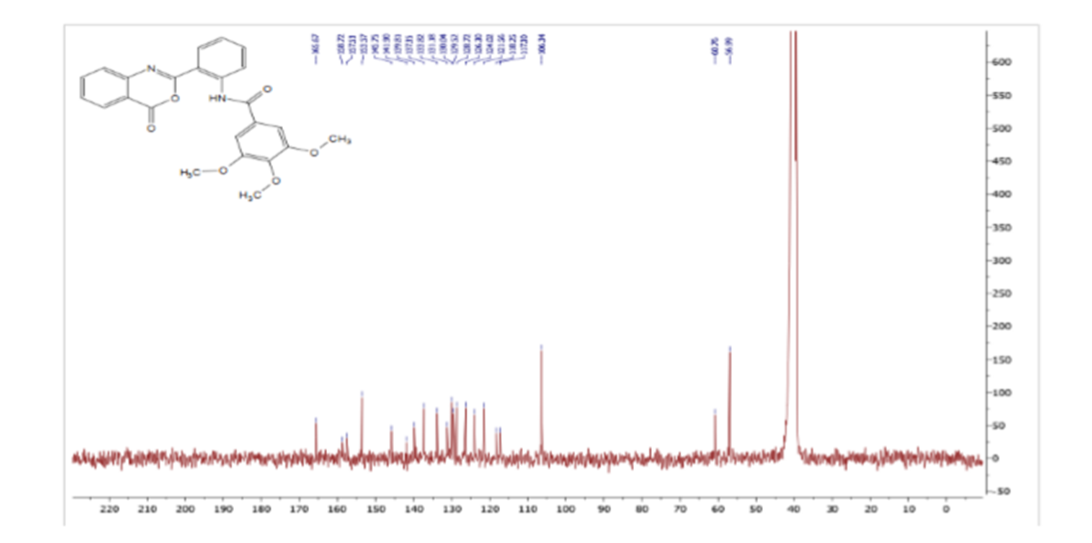
**Figure S6913CNMR spectrum of 6j**

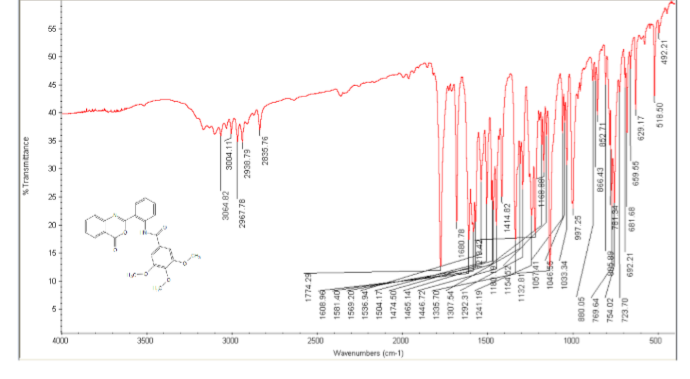
**Figure S70FT-IR spectrum of 6j**



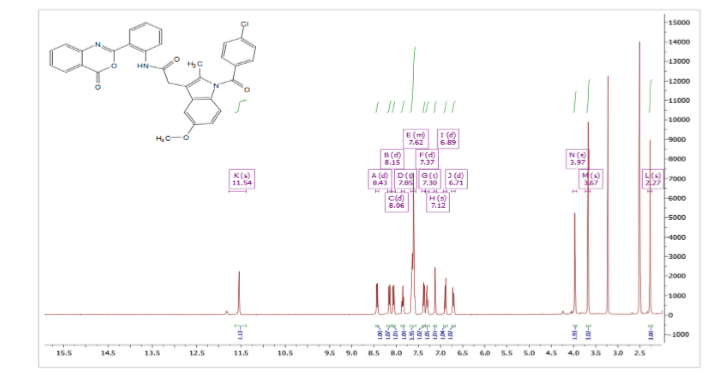
**Figure S71 1H NMR spectrum of 6k**

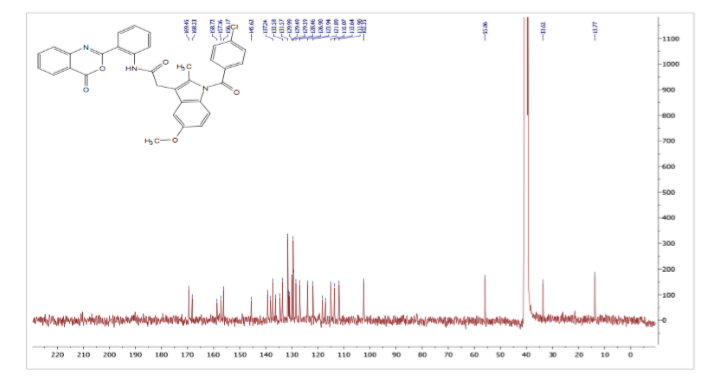
**Figure S72 13CNMR spectrum of 6k**

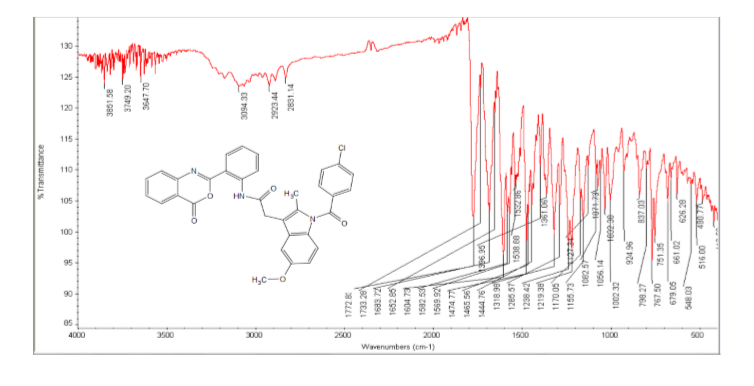


**Figure S73 FT-IR spectrum of 6k**

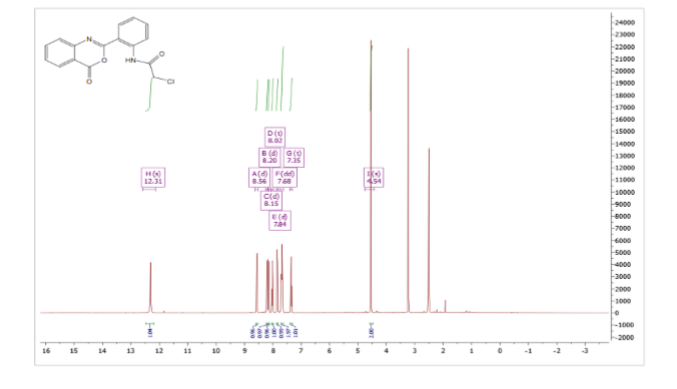
**Figure S74 1H NMR spectrum of 6l**



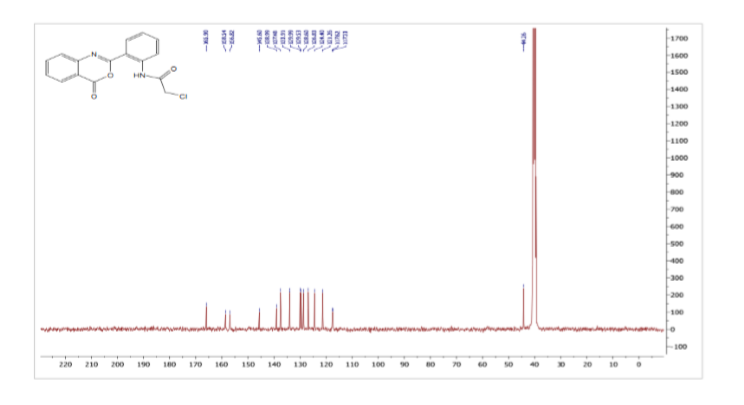
**Figure S75 13CNMR spectrum of 6l**

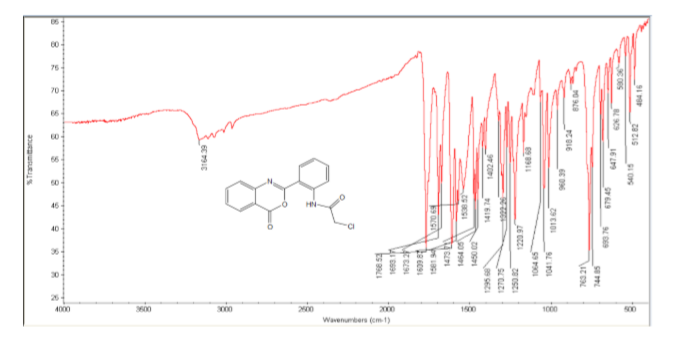
**Figure S76 FT-IR spectrum of 6l**

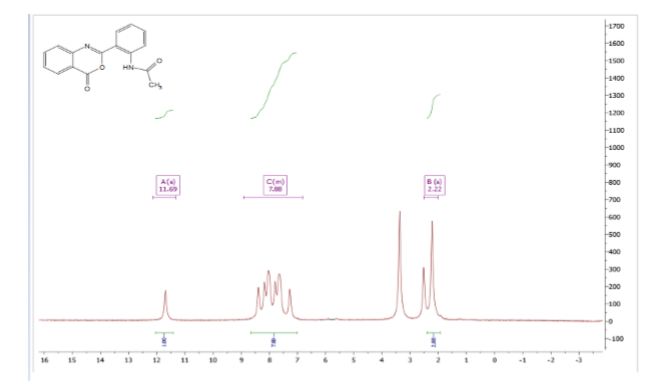
**Figure S77 1H NMR spectrum of 6m**



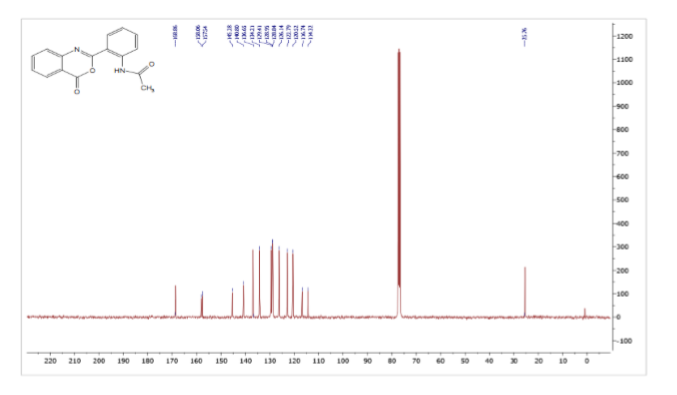
**Figure S78 13CNMR spectrum of 6m**



**Figure S79 FT-IR spectrum of 6m**

**Figure S80 1H NMR spectrum of 6n**

**Figure S8113CNMR spectrum of 6n**



Enzymatic HIV-1 RTI Assay S1*. 20*

Recombinant HIV-1-RT (4–6 ng) diluted in lysis buffer (20 µL per well) was used. In a separate reaction, lysis buffer with no HIV-1-RT was used as a negative control. Then, 20 µL of RT inhibitor diluted in lysis buffer was incubated with 20 µL of reaction mixture per reaction tube for 1 h at 37 °C. Sufficient foil bags for the number of MP modules were opened and used. The MP modules are then put into the frame in the correct orientation. MP modules were deemed as ready to be used and did not need to be rehydrated prior to the addition of the samples. The samples (60 µL) were transferred into the wells of the MP modules and covered with a cover foil before being incubated for 1 h at 37 °C. The solution was then completely removed before each well was rinsed five times with 250 µL of washing buffer for 30 s, which was then carefully removed. Diluted anti-IG-POD (200 µL, 200 Mu/mL) was added to each well; then, the MP modules were covered with a cover foil and incubated for 1 h at 37 °C. The solution was then completely removed, and each well was rinsed five times using 250 µL of washing buffer for 30 s, which was then carefully removed. ABTS substrate solution (200 µL) was added to each well, which was then incubated at +15 °C to +25 °C until the color of the solutions (green color) was sufficient for photometric detection (10–30 min). The absorbance of the samples was measured using a microplate (ELISA) reader, at 450 nm (reference wavelength of approximately 490 nm).

**Cytotoxicity Evaluation, CE S221**

***Assay Procedure*-** was carried out using viability assay. For cytotoxicity assay, the cells were seeded in a 96-well plate at a cell concentration of 1 × 104 cells per well in 100 µL of growth medium. A fresh medium containing different concentrations of the test sample was added after 24 h of seeding. Serial twofold dilutions of the tested chemical compounds were added to confluent cell monolayers dispensed into 96-well, flat-bottomed microtiter plates (Falcon, NJ, USA) using a multichannel pipette. The microtiter plates were incubated at 37 °C in a humidified incubator with 5% CO2 for a period of 24 h. Three wells were used for each concentration of the test sample. Control cells were incubated without a test sample and with or without dimethyl sulfoxide (DMSO). The small percentage of DMSO present in the wells (maximum of 0.1%) was found not to affect the experiment. After incubation of the cells at 37 °C for 24 h, the viable cell yield was determined using a colorimetric method.

In brief, after the end of the incubation period, the solvent media were aspirated and crystal violet solution (1%) was added to each well for at least 30 min. The stain was removed, and the plates were rinsed using tap water until all excess stain was removed. Glacial acetic acid (30%) was then added to all of the wells and mixed in thoroughly, and then, the absorbance of the plates was measured after gentle shaking on a microplate reader (TECAN, Inc.) at a test wavelength of 490 nm. All results were corrected for background absorbance detected in wells without added stain. Treated samples were compared with the cell control in the absence of the tested compounds. All experiments were carried out in triplicate. The cell cytotoxicity effects of each tested compound were calculated. The optical density was measured using a microplate reader (Sunrise, TECAN, Inc., USA) to determine the number of viable cells, and the percentage of viability was calculated using [(ODt/ODc)] × 100%, where ODt is the mean optical density of wells treated with the test samples and ODc is the mean optical density of untreated cells. The relationship between the surviving cells and drug concentration was plotted to obtain the survival curve of each tumor cell line after treatment with the specified compound. The 50% inhibitory concentration (IC50), the concentration required to cause toxic effects in 50% of intact cells, was estimated from plots of the dose-response curve for each concentration using GraphPad Prism software (San Diego, CA, USA).

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