**Design, Synthesis and Evaluation of Novel Phenanthridine Triazole Analogs as Potential Antileishmanial Agents**

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**Experimental section**

***1. Materials and methods***

All chemical reagents and solvents are purchased from Aldrich, Alfa Aesar, Finar. The solvents and reagents were of LR grade. All the solvents were dried and distilled before use. Thin-layer chromatography (TLC) was carried out on aluminium-supported silica gel plates (Merck 60 F254) with visualization of components by UV light (254 nm). Column chromatography was carried out on silica gel (Merck 100-200 mesh). 1H NMR and 13C NMR spectra were recorded at 400 MHz and 101 MHz respectively using a Bruker AV 400 spectrometer (Bruker CO., Switzerland) in CDCl3 and DMSO-*d6* solution with tetramethylsilane as the internal standard and chemical shift values (*δ*) were given in ppm. 1H NMR spectra were recorded in CDCl3 or DMSO-*d6*. The following abbreviations are used to designate multiplicities: s = singlet, d = doublet, t = triplet, m = multiplet, br = broad. Melting points were determined on an electro thermal melting point apparatus (Stuart-SMP30) in open capillary tubes and are uncorrected. Elemental analyses were performed by ElementarAnalysensysteme GmbH vario MICRO cube CHN Analyzer. Mass spectra (ESI-MS) were recorded on Schimadzu MS/ESI mass spectrometer.

**1.1** **General procedure for synthesis of compounds T01-T14**

**Synthesis of compound T02:**

Compound **T01** (1 eq.) was taken in dioxane then added ethyl 2-chloroacetoacetate (3 eq.) at rt. The reaction mixture was heated to 100 ℃ and allowed for stirring overnight. The reaction was monitored by TLC. After completion of reaction, the total reaction mixture was extracted with excess ethyl acetate. The organic layers were washed with brine solution, separated and dried over anhydrous sodium sulphate and evaporated under reduced pressure. The crude product was purified with silica gel column chromatography by eluting with 0- 20% ethyl acetate and petroleum ether. The compound **T02** was obtained in 79% yield. Structure of **T02** was confirmed with mass and NMR spectras.

**Synthesis of compound T03:**

Compound **T02** (1 eq.) was taken in ethanolic water mixture then LiOH (5 eq.) was added at rt. The total reaction mixture was heated to 80 ℃ and stirred for 5 h. The reaction was monitored by TLC. After completion of reaction, the reaction mixture was evaporated under reduced pressure. The crude product was washed with ethyl acetate (2X). The obtained compound was dissolved in water, and pH was adjusted to 7 by the addition of saturated citric acid. The precipitated compound was filtered under vacuum. The compound **T03** was obtained in 75% yield confirmed with mass and NMR spectras.

**Synthesis of compound T04:**

Compound **T03** (1 eq.) was taken in DMF then DIPEA (3 eq.) was added at rt. To this reaction mixture HOBT (1.5 eq.), EDC.HCl (1.5 eq.) and then propargylamine (1.1 eq.) was added. The mixture was stirred at rt for 5 h. The reaction was monitored by TLC. After completion of reaction, the reaction mixture was extracted with ethyl acetate. The organic layer was dried over anhydrous sodium sulphate and evaporated under reduced pressure. The crude product was purified with silica gel column chromatography by eluting with 0-50% ethylacetae: petroleum ether. The compound **T04** is obtained in 80% yield and confirmed with mass and NMR spectras.

**Synthesis of Compounds T05-T14:**

Compound **T04** (1 eq.) was taken in DMF: t-BuOH: water then CuSO4 (5 mol %), CuI (5 mol %), sodium ascorbate (1.5 eq.) was added at rt. To this reaction mixture substituted azides (1.1 eq.) were added and stirred at rt for 5 h. The reaction was monitored by TLC. After completion of reaction, the total reaction mixture was extracted with ethyl acetate. The organic layer was dried over anhydrous sodium sulphate and evaporated under reduced pressure. The crude product was purified with silica gel column chromatography by eluting with 0-100% ethylacetae: pet ether. The triazoles **T05-T14** were confirmed with mass and NMR data.

**Table S1.** Crystal data and refinement parameters for compounds **T01** and **T09**

|  |  |  |
| --- | --- | --- |
| **Identification Code** | **T01** | **T09** |
| Empirical formula | C13H10N2 | C28H23Cl2N7O4 |
| Formula weight | 194.238 | 592.444 |
| Temperature/K | 100 | 100 |
| Crystal system | monoclinic | triclinic |
| Space group | P21/n | P-1 |
| a/Å | 13.2447(10) | 9.5639(2) |
| b/Å | 5.0841(4) | 11.4740(2) |
| c/Å | 14.1024(11) | 14.1042(3) |
| α/° | 90 | 103.5137(16) |
| β/° | 99.197(7) | 104.7417(17) |
| γ/° | 90 | 108.6692(18) |
| Volume/Å3 | 937.41(13) | 1331.51(5) |
| Z | 4 | 2 |
| ρ calc g/cm3 | 1.376 | 1.478 |
| μ/mm‑1 | 0.649 | 2.620 |
| F(000) | 409.2 | 615.4 |
| Crystal size/mm3 | 0.1 × 0.05 × 0.03 | 0.1 × 0.08 × 0.05 |
| Radiation | Cu Kα (λ = 1.54184) | Cu Kα (λ = 1.54184) |
| 2Θ range for data collection/° | 8.5 to 159.3 | 8.66 to 159.22 |
| Index ranges | -15 ≤ h ≤ 16, -6 ≤ k ≤ 4, -17 ≤ l ≤ 17 | -11 ≤ h ≤ 11, -10 ≤ k ≤ 14, -17 ≤ l ≤ 16 |
| Reflections collected | 4175 | 15114 |
| Independent reflections | 1975 [Rint = 0.0429, Rsigma = 0.0588] | 5599 [Rint = 0.0306, Rsigma = 0.0357] |
| Data/restraints/parameters | 1975/0/137 | 5599/0/372 |
| Goodness-of-fit on F2 | 1.024 | 1.028 | |
| Final R indexes [I>=2σ (I)] | R1 = 0.0800, wR2 = 0.2298 | R1 = 0.0497, wR2 = 0.1446 |
| Final R indexes [all data] | R1 = 0.1016, wR2 = 0.2499 | R1 = 0.0520, wR2 = 0.1466 |

***2. Biological procedures***

***2.1. In-vitro* antileishmanial activity**

Anti-leishmanial activity of the compounds were assayed *in vitro* against *Leishmania infantum* BCN150 iRFP promastigotes, a genetically modified strain with iRFP for infrared detection (Calvo-Álvarez et al., 2012). Promastigotes were cultured in M199 medium (Gibco), supplemented with 25 mM HEPES pH 6.9, 7.6 mM hemin, 10 mM glutamine, 0.1 mM adenosine, 0.01 mM folic acid, 1xRPMI 1640 vitamin mix (Sigma), 10% (v/v) heat inactivated foetal bovine serum (Gibco) and antibiotic mixture. *L. infantum* promastigotes cultures with a density of 1 x 106 cells/mL, were aliquoted into 96-well optical bottom black plates (Thermo Scientific), 180 µL per well. Each compound was tested adding 20 µL of different stock solutions to wells. Stock solutions were prepared in DMSO and stepwise diluted in M199 media (0.07-300 µM). Viability of promastigotes was assessed measuring their fluorescence at 708 nm in an Odyssey (Li-Cor) infrared imaging system. All compounds and controls were assayed by triplicate.

***2.2. Preparation and assay of bone marrow axenic amastigotes***

***Ex vivo* murine splenic explant cultures (intramacrophage amastigote assay)**

Primary cultures of infected splenic explants were used to assess the *ex vivo* effect of compounds **T01** and **T08** on the intramacrophage stage of the parasite. For this purpose, 108 iRFP-*L. infantum* metacyclic promastigotes were inoculated intraperitoneally to female BALB/c mice. After 5 weeks, mice were humanely sacrificed and spleens were dissected, washed with cold phosphate-buffered saline (PBS), cut in small pieces and incubated with 5 mL of 2 mg/mL collagenase D (Sigma) prepared in buffer (10mM HEPES, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl2 and 1.8 mM CaCl2) for 20 min, to obtain a cell suspension. The cell suspension was passed through a 100 μm-mesh cell strainer, harvested by centrifugation (500×g for 7 min at 4 °C), washed twice with PBS and re-suspended in RPMI medium (Gibco), supplemented with 10 mM HEPES, 1 mM sodium pyruvate, 1xRPMI 1640 vitamin mix, 10% (v/v) FBS, and antibiotics. Similar procedure was applied to obtain uninfected splenic cultures in order to determine selectivity index of the compounds. Different concentrations of the tested compounds (1.25, 2.5, 5.0 and 10 μM) were added to these cultures seeded in 384-well black optical bottom plates at 37 °C under 5% CO2 atmosphere. The viability of iRFP-*L. infantum* amastigotes infecting macrophages was registered daily by recording the emitted fluorescence at 713 nm by an Odyssey (Li- Cor) infrared imaging system.

***2.3. Cytotoxicity assay***

The safety of the compounds was tested both on primary cultures of murine splenocytes and on human hepatocarcinoma cell HepG2 line (ATCC HB-8065) as a suitable *in vitro* toxicity model system of human hepatocytes. HepG2 cells and splenocyte cells were seeded in 96-well plates at 37ºC under 5% CO2 atmosphere. For HepG2 cells was used The Glutamax Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco), supplemented with 10% (v/v) FBS, and antibiotic mix. Viability of splenocytes and hepatocarcinoma cells were measured using the Almar Blue staining method, according to manufacturer’s recommendations (Invitrogen). Selectivity index for each compound was calculated as the ratio between the CC50 value obtained for non-infected murine splenocytes or CC50 value obtained for HepG2 cells and the EC50 values for promastigotes/amastigotes obtained with ex vivo murine splenic explant cultures.

***3. Computation studies***

***3.1. In-silico prediction of ADME and Toxicity (ADMET) parameters***

TheADMET parameters of the titled compounds were *in silico* predicted using Qikprop module of Schrodinger. The diverse parameters predicted were molecular weight (M.Wt.), total solvent accessible surface area (SASA), number of hydrogen bond donor (HBD), number of hydrogen bond acceptor (HBA), octanol / water partition coefficient (log P), aqueous solubility (Log S), predicted apparent Caco-2 cell permeability in nm/sec (P Caco) and number of rotatable bonds (Rot) 2,3. SMILES format of the compounds was generated by using OSIRIS DataWarrior. All the related toxicity parameters were also predicted by the same software 4. The ideal ranges, which are followed by 95% of the approved drugs, are discussed in supplementary information (**Table S2**)

**Table S2.** Optimum range of drug-likeness parameters followed by 95% of approved drugs

|  |  |  |
| --- | --- | --- |
| **S. No** | **Molecular descriptor** | **Optimum range** |
| 1 | Molecular weight (Mol wt.) | 130-725 |
| 2 | Total solvent accessible surface area in square angstroms (SASA) | 300-1000 |
| 3 | No. of hydrogen bond donor groups (HBD) | 0- 6 |
| 4 | No. of hydrogen bond acceptor groups (HBA) | 2 to 20 |
| 5 | Octanol/water partition coefficient (LogP) | -2.0 to 6.5 |
| 6 | Aqueous solubility, in mol/l (logS) | –6.5 to 0.5 |
| 7 | Apparent Caco-2 cell permeability in nm/s (PCaco) | <25 poor, > 500 great |
| 8 | Brain/blood partition coefficient (logBB) | –3.0 to 1.2 |
| 9 | No. of rotatable bonds (Rot) | 0 to 15 |

***3.2. Molecular docking study***

Molecular docking study was carried out using Schrodinger software 5 (Version 2019-1, Schrodinger) installed on Intel Xenon W 3565 processor and Ubuntu enterprise version 14.04 as an operating system. The selected target protein structure was retrieved from the RCSB protein data bank ([www.rcsb.org](http://www.rcsb.org)) 6. Targeted ligands were drawn using ChemDraw 18.0 software.

***3.2.1. Ligand preparation***

The ligands used as an input for docking study was sketched using ChemDraw software and cleaned up the structures for bond alignment, ligands incorporated into the workstation, the energy was minimized using OPLS3e force field in Ligprep 7 (Version 2019-1, Schrodinger). This minimization helps to assign bond orders, the addition of hydrogens to the ligands, and conversion of 2D to 3D structure for further docking studies. The generated output file (best conformations of the ligands) was further used for docking studies.

***3.2.2. Protein preparation***

Protein was retrieved from the RCSB site (<https://www.rcsb.org/structure/2JK6> ) 8. Protein was prepared using a protein preparation wizard 9 (Version 2019-1, Schrodinger). Hydrogen atom was added to the proteins, and charges were assigned. Generated Het states using Epik at pH 7.0 ±2.0. Pre-processed the protein and refined, modified the protein by analyzing the workspace, water molecules, and other heteroatoms were examined, non-significant atoms were excluded from the crystal structure of the protein. Finally, the protein was minimized by using OPLS3e force filed

***3.2.3. Receptor grid generation***

A receptor grid was generated around the protein by picking the inhibitory ligand (X-ray pose of the ligand in the protein). The centroid of the ligand was selected to create a grid box around it, and the Vander Waals radius of receptor atoms was scaled to 1.00 Å with a partial atomic charge of 0.25.

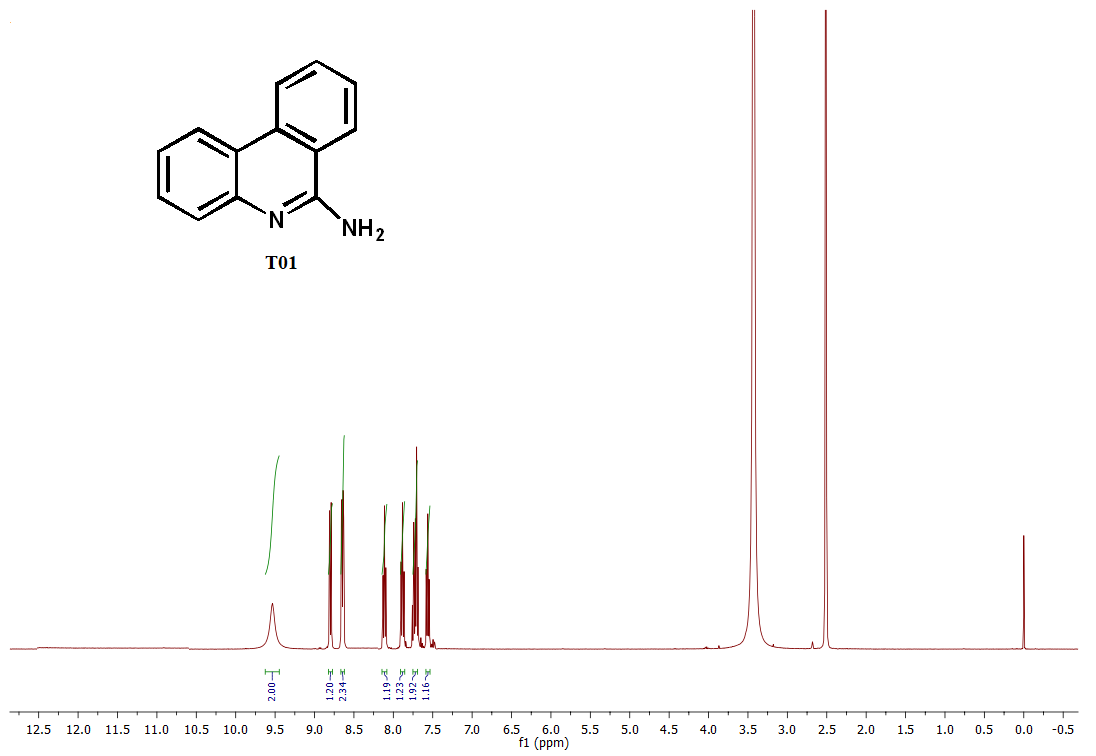
***3.2.4. Docking validation***

The most straightforward way of validating the accuracy of specified parameters for docking study is to re-dock the co-crystallized ligand back into the binding site of the protein and calculate the root mean square deviation (RMSD) value between the crystallographic orientation and the docked pose. RMSD calculation is a convenient method to use in order to follow how much a structure has diverged from its initial geometry. The lower the RMSD value between the docked pose to that of its crystallographic orientation is an indication of the suitability of the docking protocols. Therefore, prior to screening of all ligands, the co-crystal structures of PDB-2JK6 (FAD molecule), was chosen and re-docked back into the same active site. The RMSD value between the crystallographic orientation and the best-docked pose was generated. The RMSD value of the selected targets was found to be 0.20 Å respectively. The lower RMSD value indicates that the docking protocol could be reliable for the final docking studies of the test compounds against the selected target.

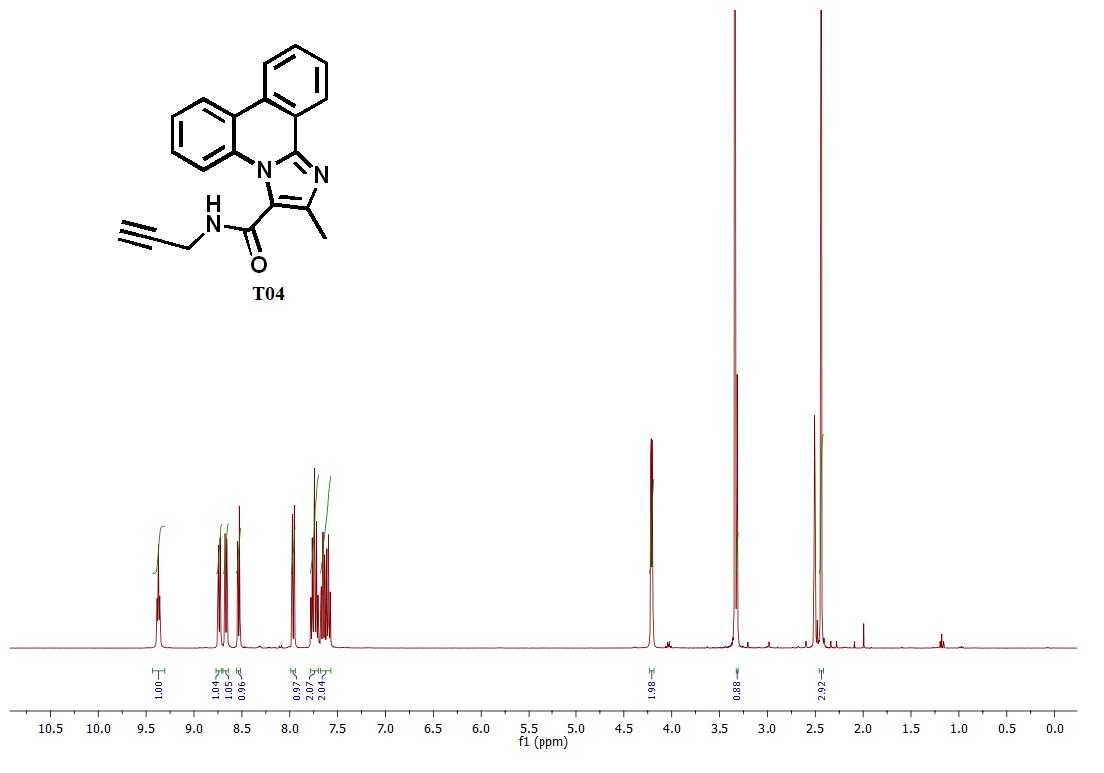
***3.2.5. Docking and analysis***

Molecular docking was performed using the above-prepared ligand and protein as input. The results of the docking study were analyzed with the help of XP Visualiser (Version 2019-1, Schrodinger). Docking studies of the designed and synthesized molecules were performed by using the Glide module 10 in Schrodinger. All docking calculations were performed using Extra Precision (XP) mode. A scaling factor of 0.8 and a partial atomic charge of less than 0.15 was applied to the atoms of the protein. Glide docking score was used to determine the best-docked confirmation from the output. The interactions of these docked conformations were investigated further using XP visualizer.

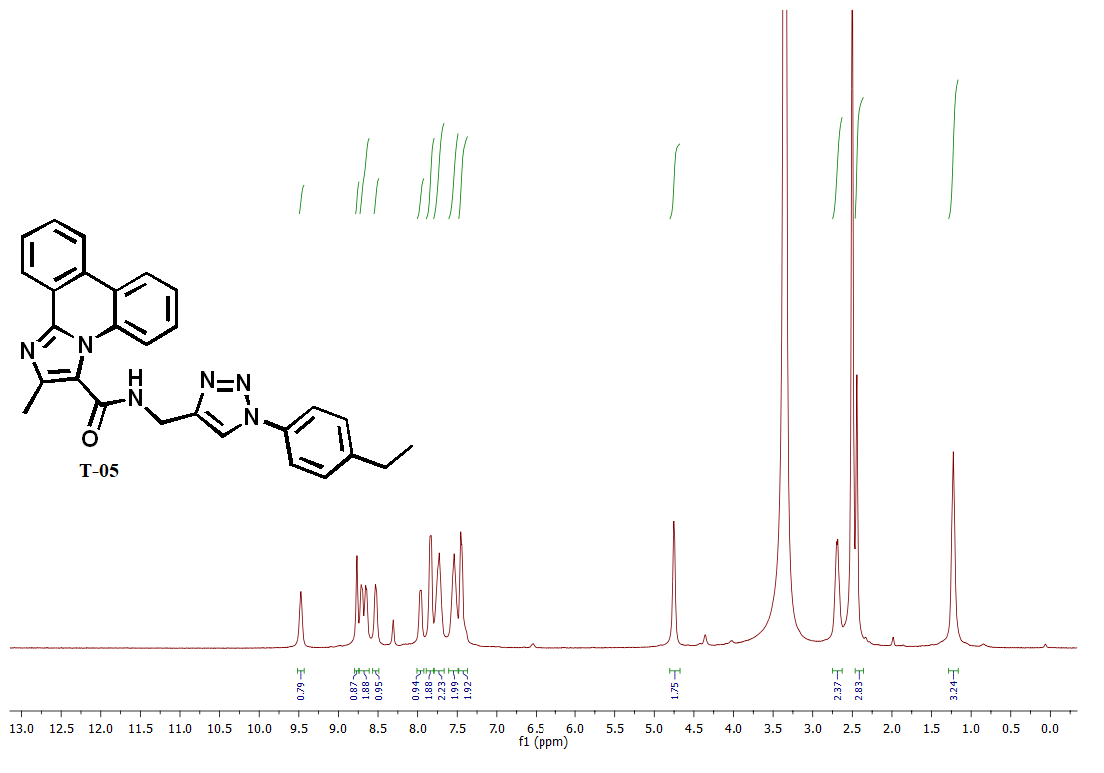
***4. 1H NMR spectras of final compounds***



**1HNMR spectrum of compound T01**

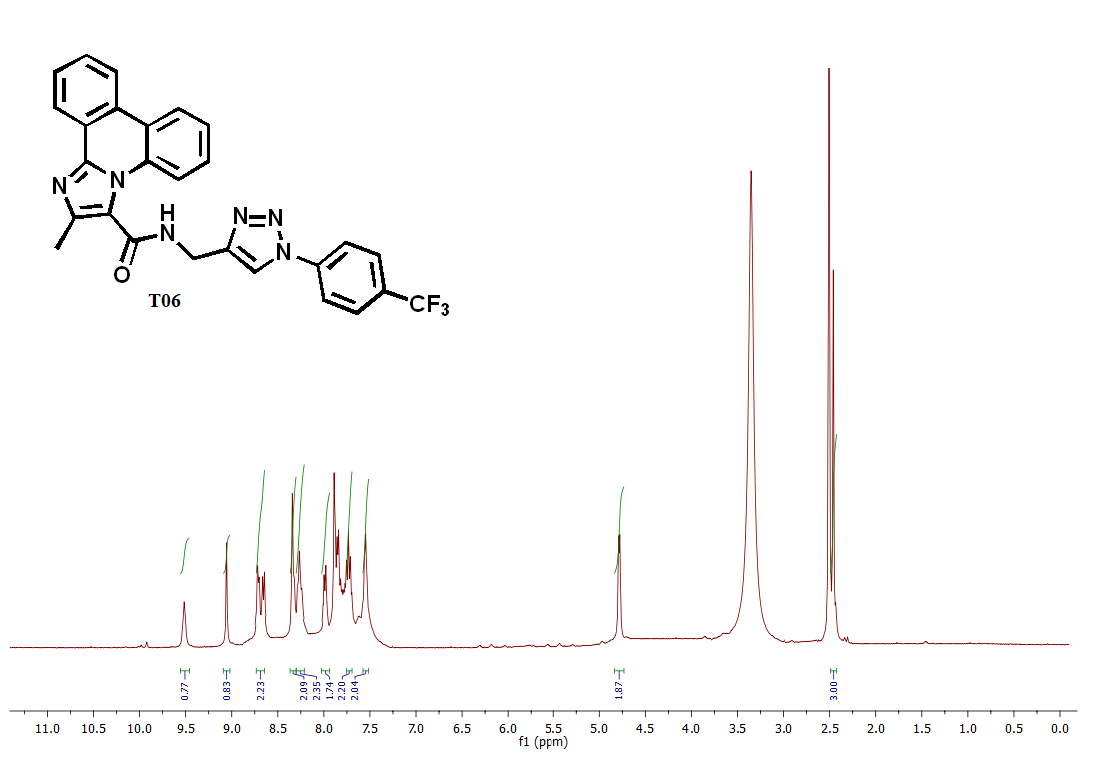


**1HNMR spectrum of compound T04**

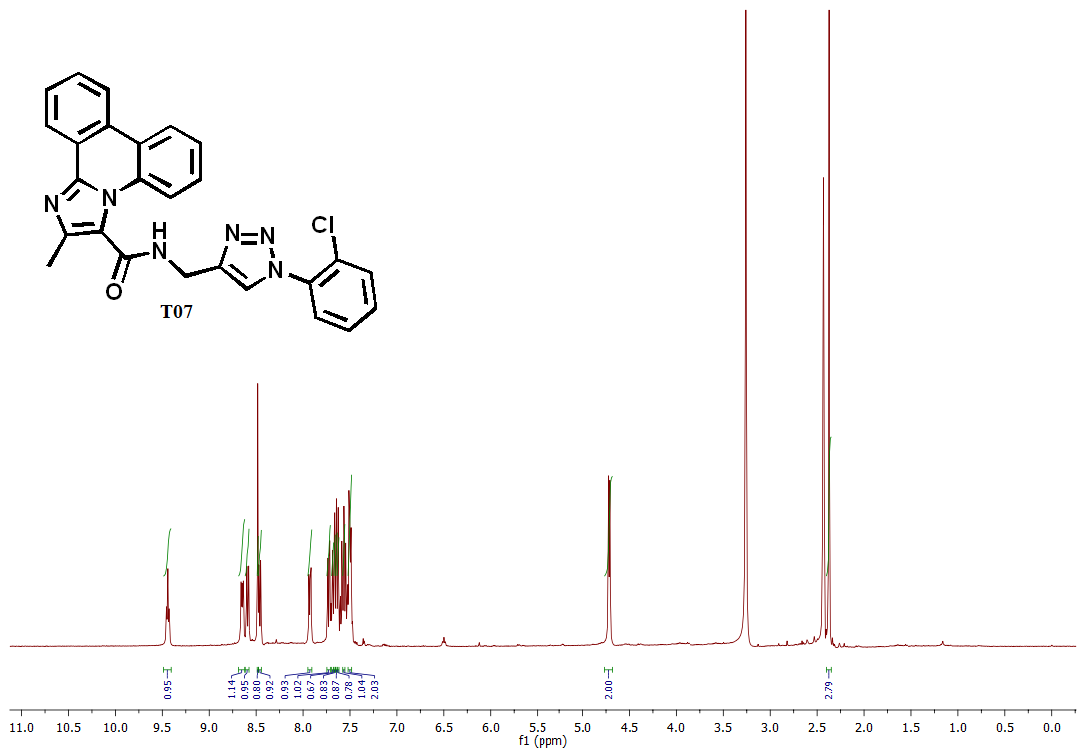


**T05**

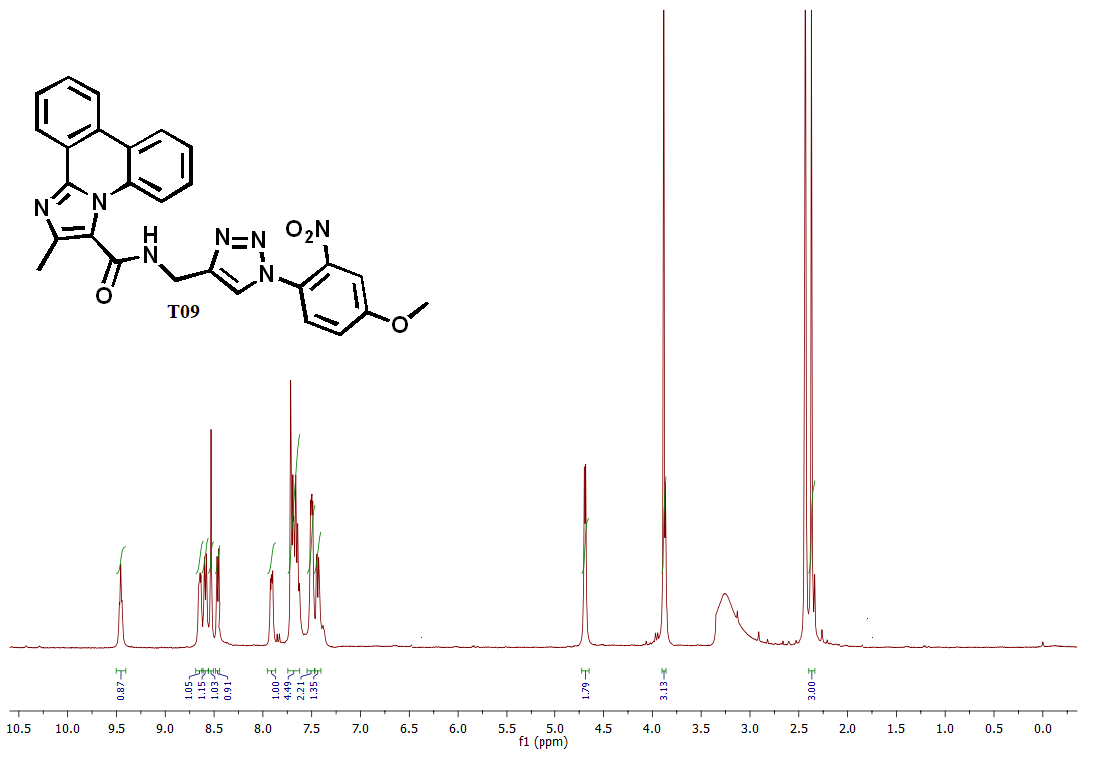
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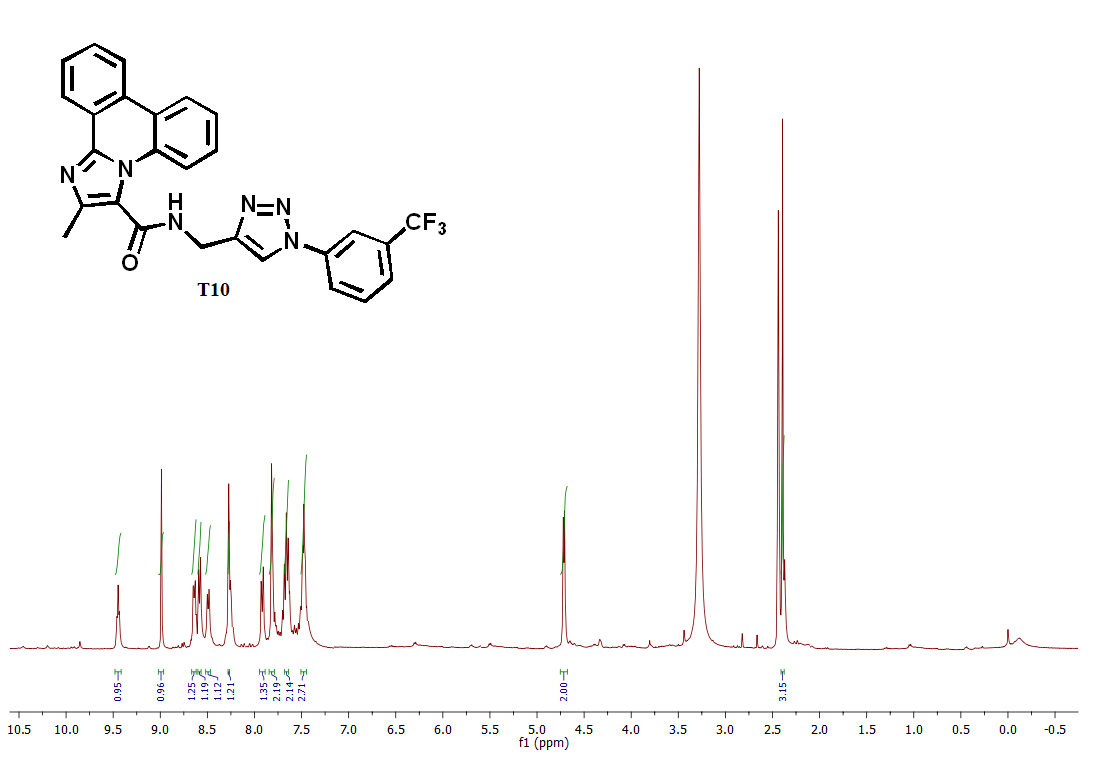
**1HNMR spectrum of compound T06**



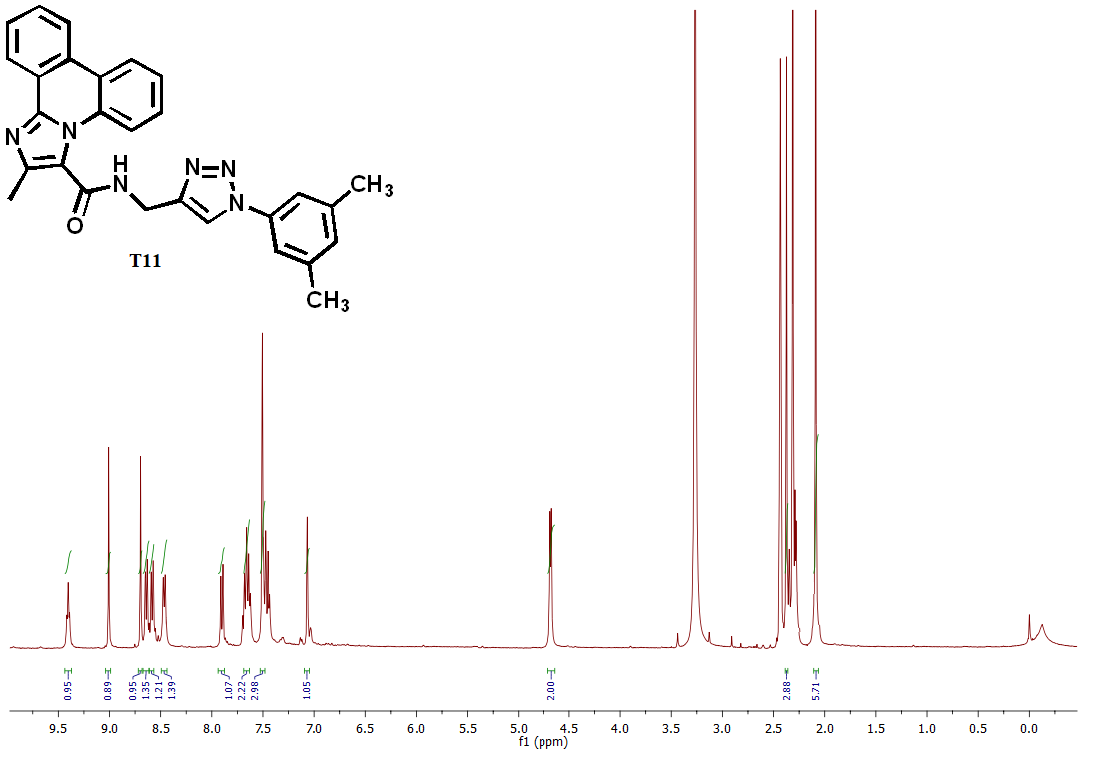
**1HNMR spectrum of compound T07**



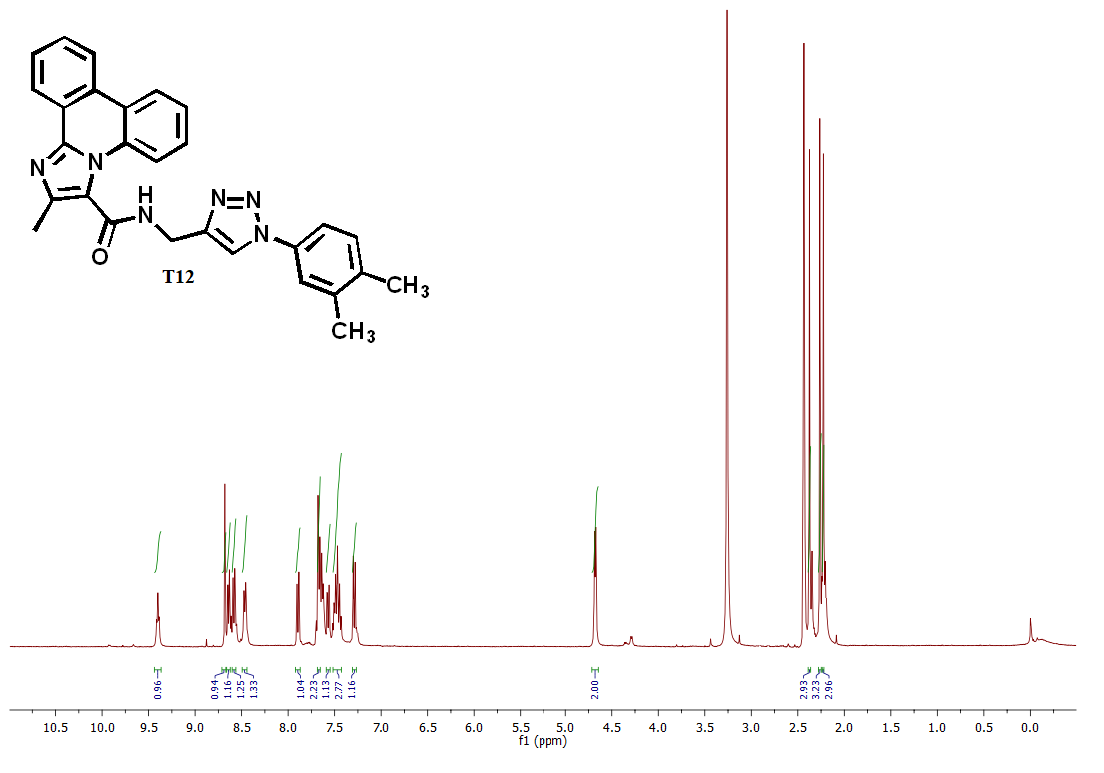
**1HNMR spectrum of compound T09**



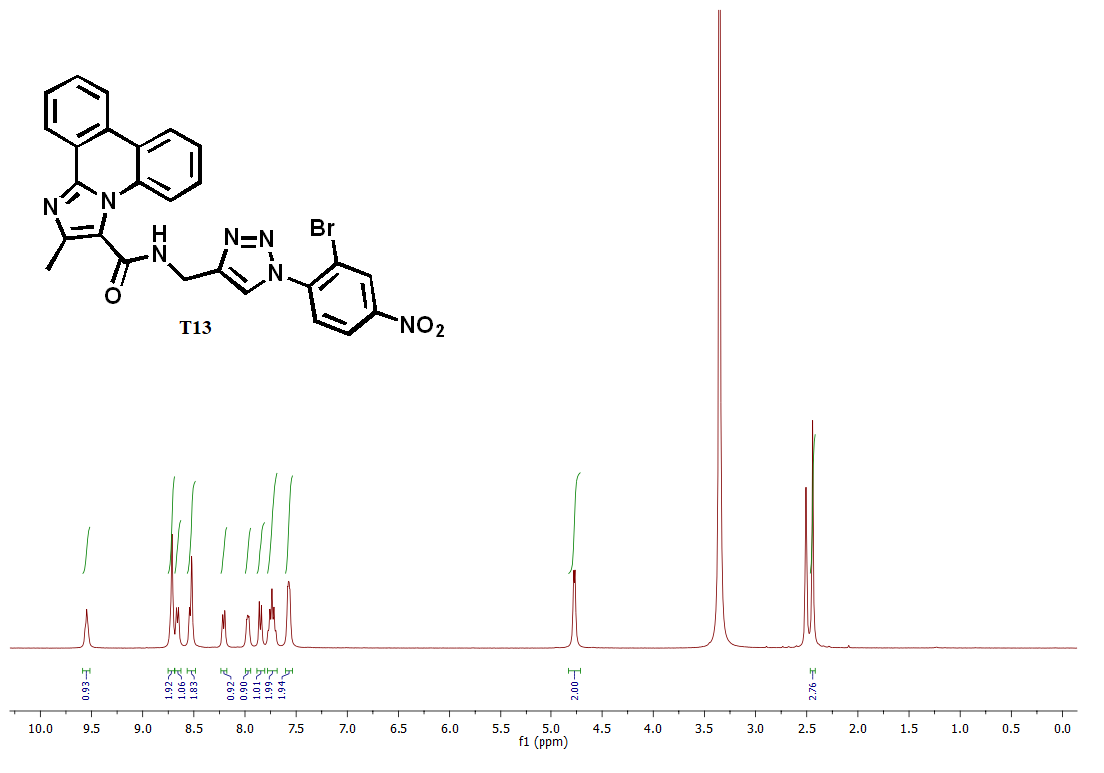
**1HNMR spectrum of compound T10**



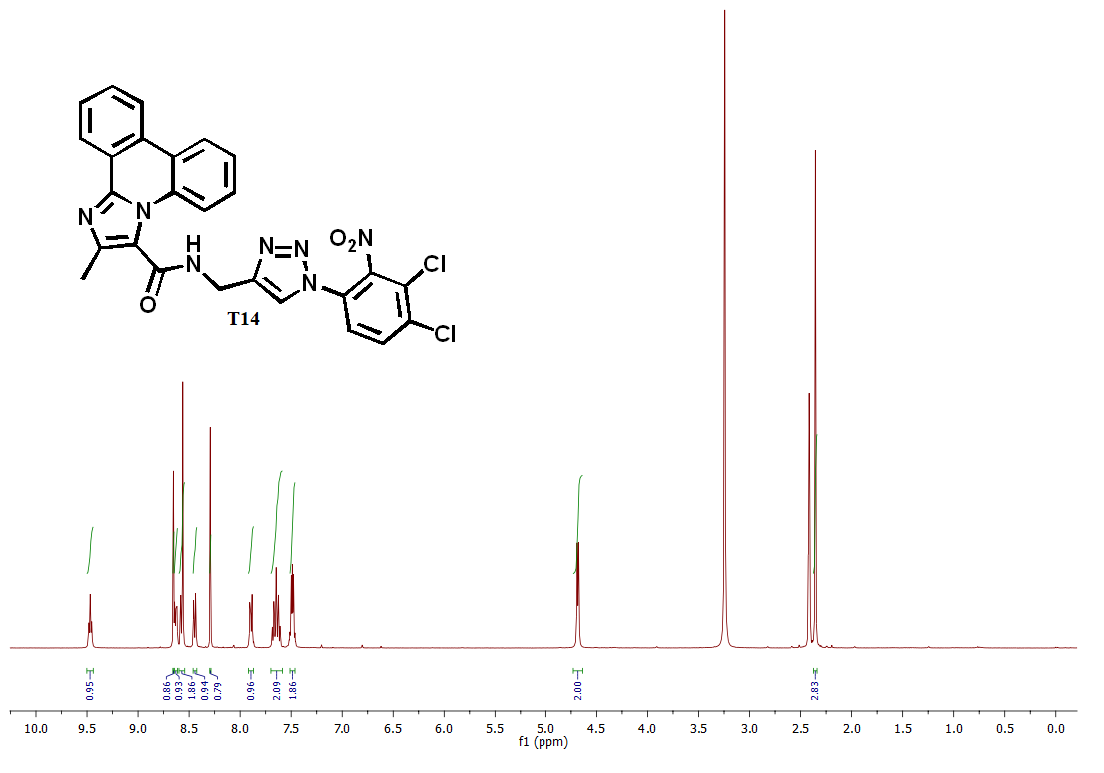
**1HNMR spectrum of compound T11**



**1HNMR spectrum of compound T12**

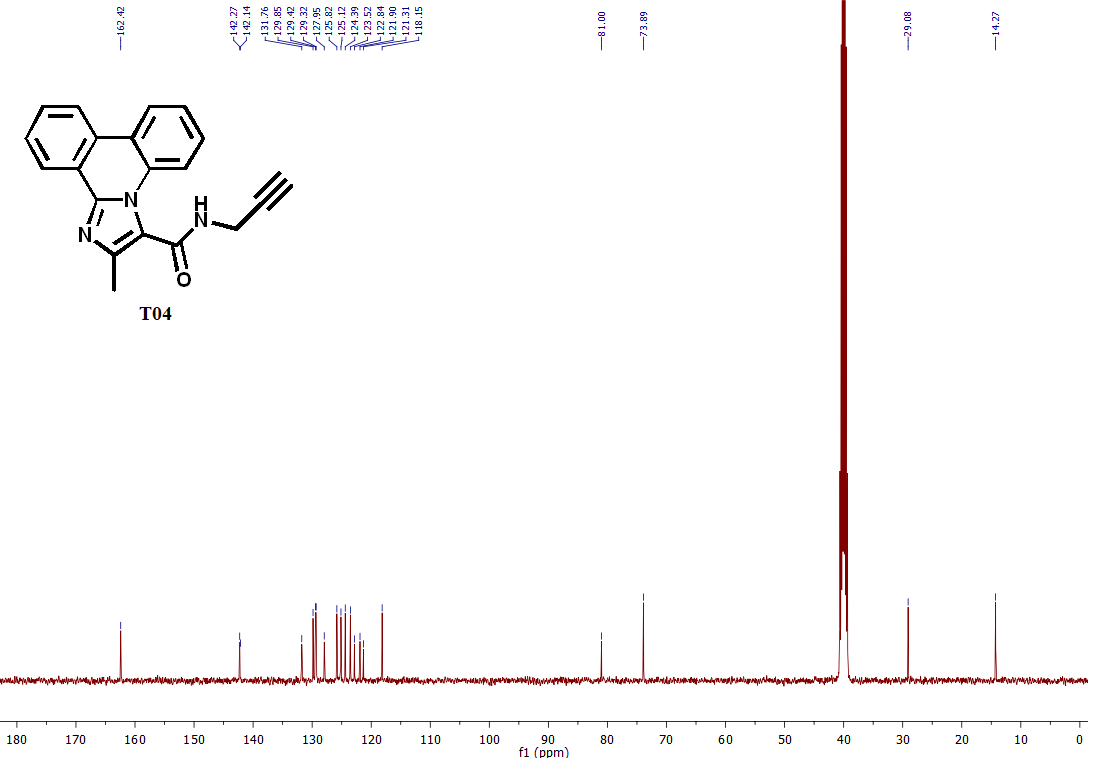


**1HNMR spectrum of compound T13**

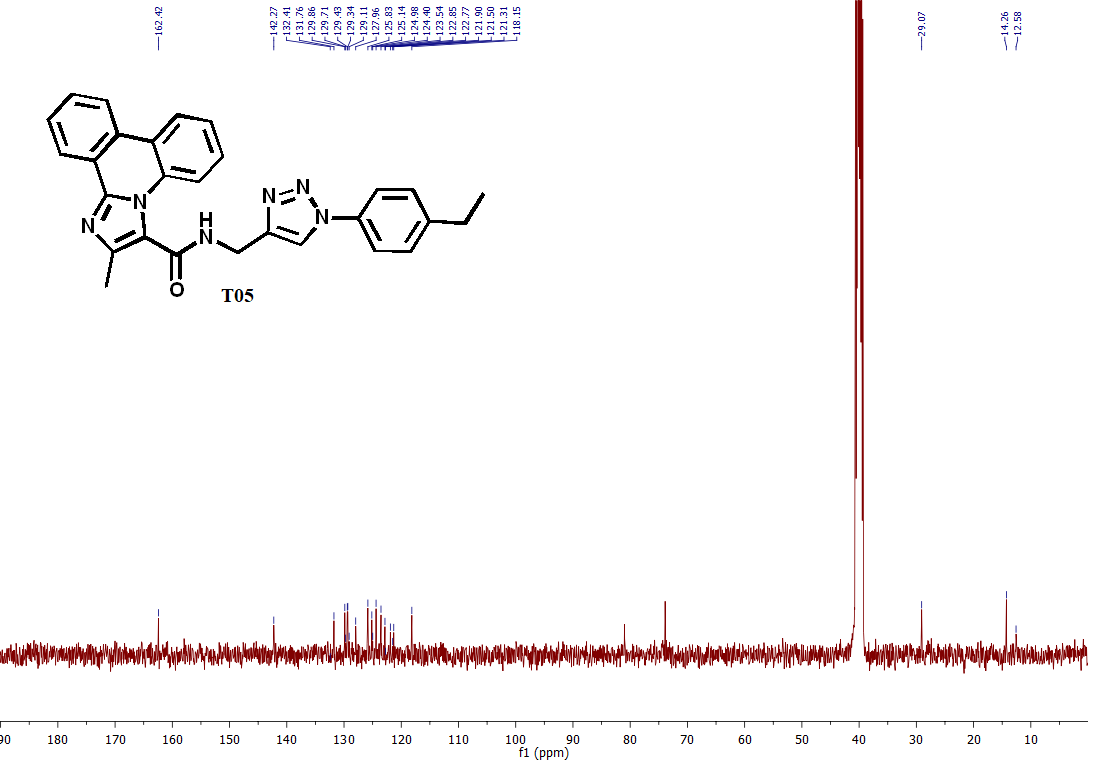


**1HNMR spectrum of compound T14**

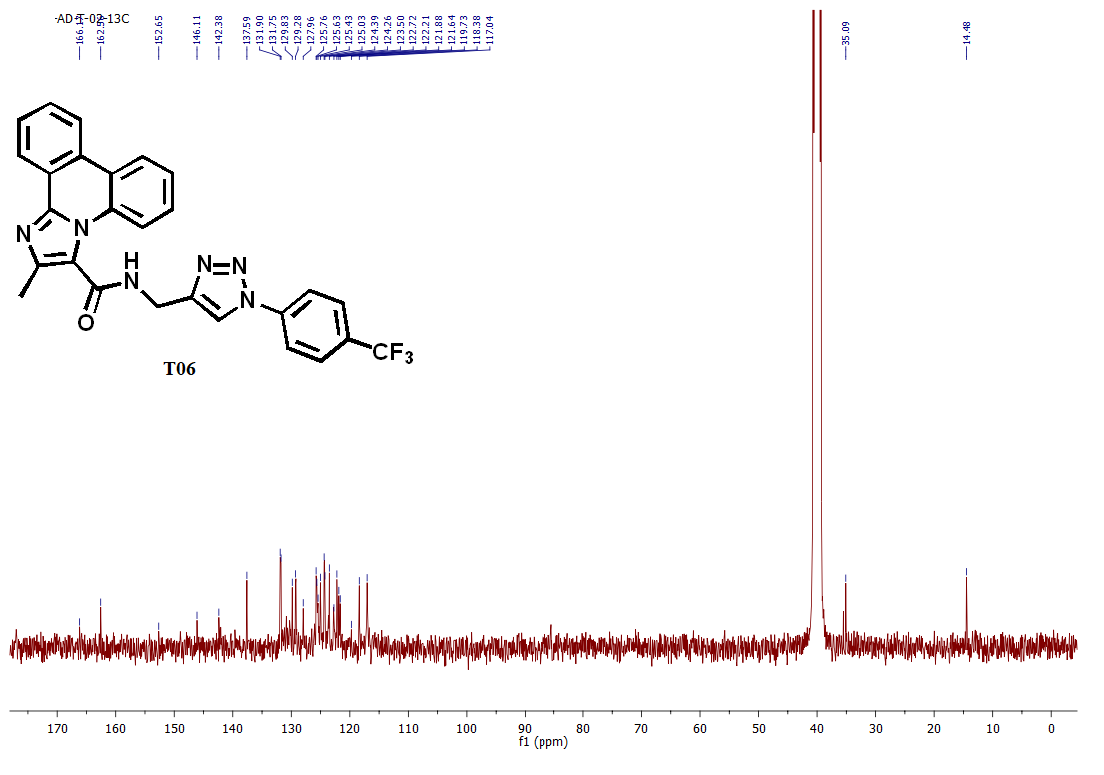
***5. 13C NMR spectras of final compounds***



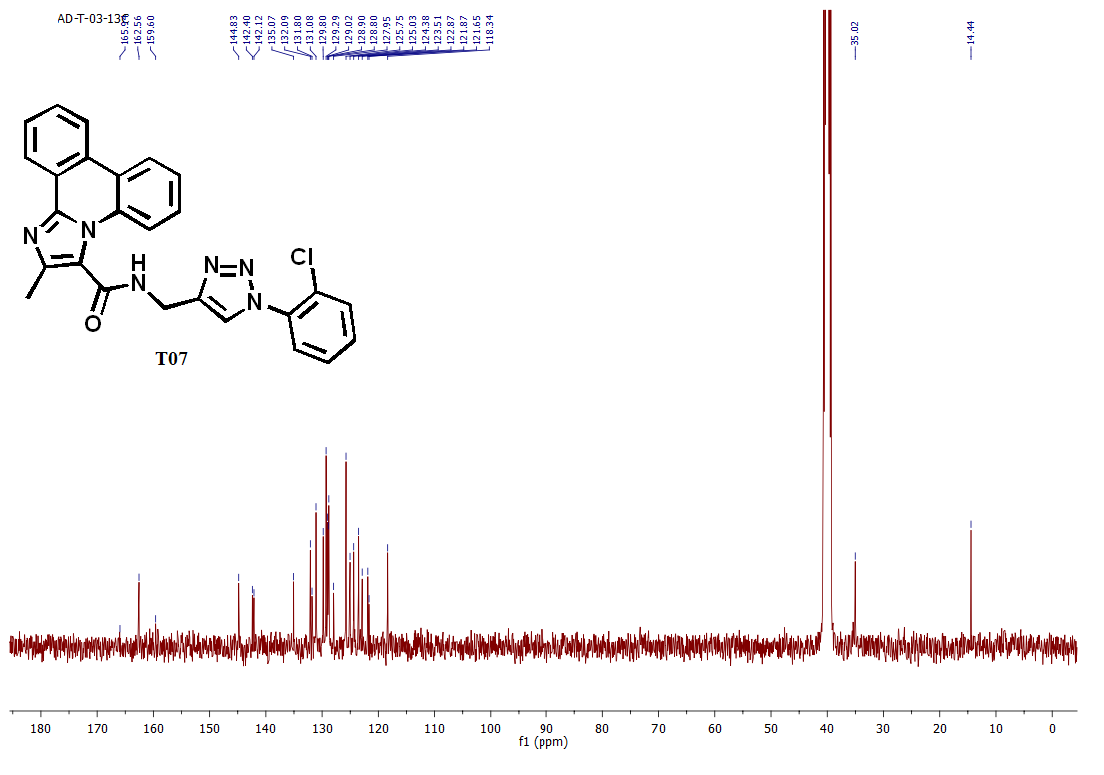
**13CNMR spectrum of compound T04**



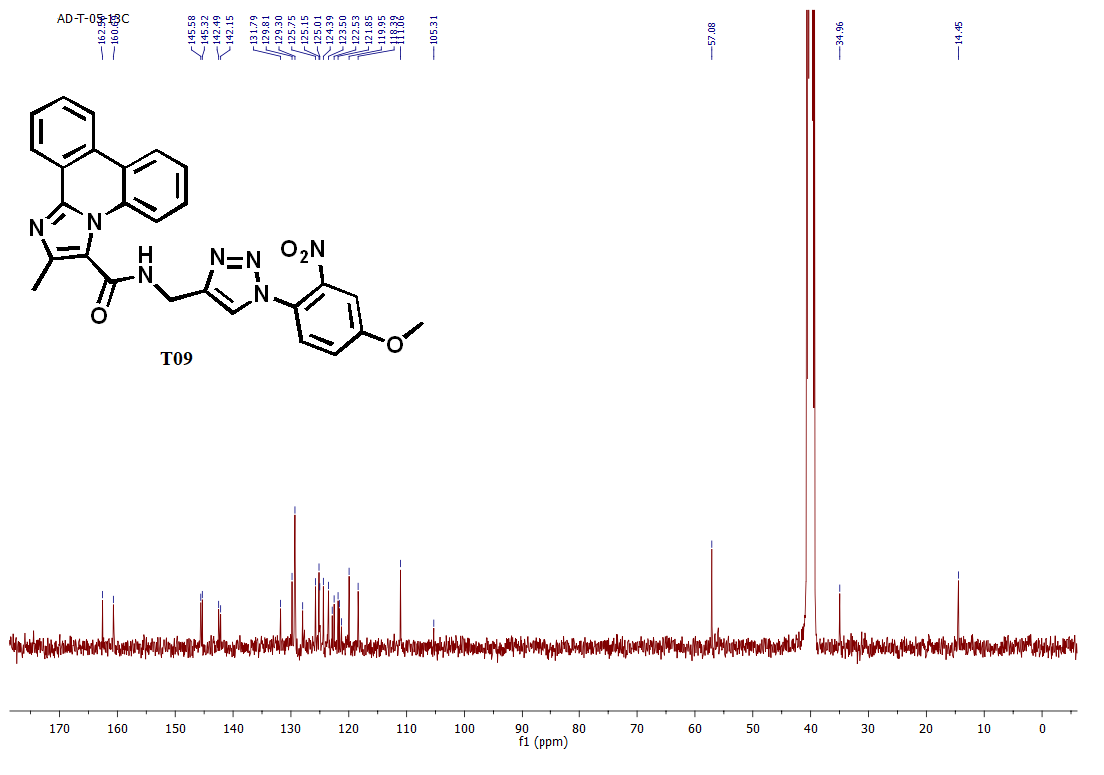
**13CNMR spectrum of compound T05**



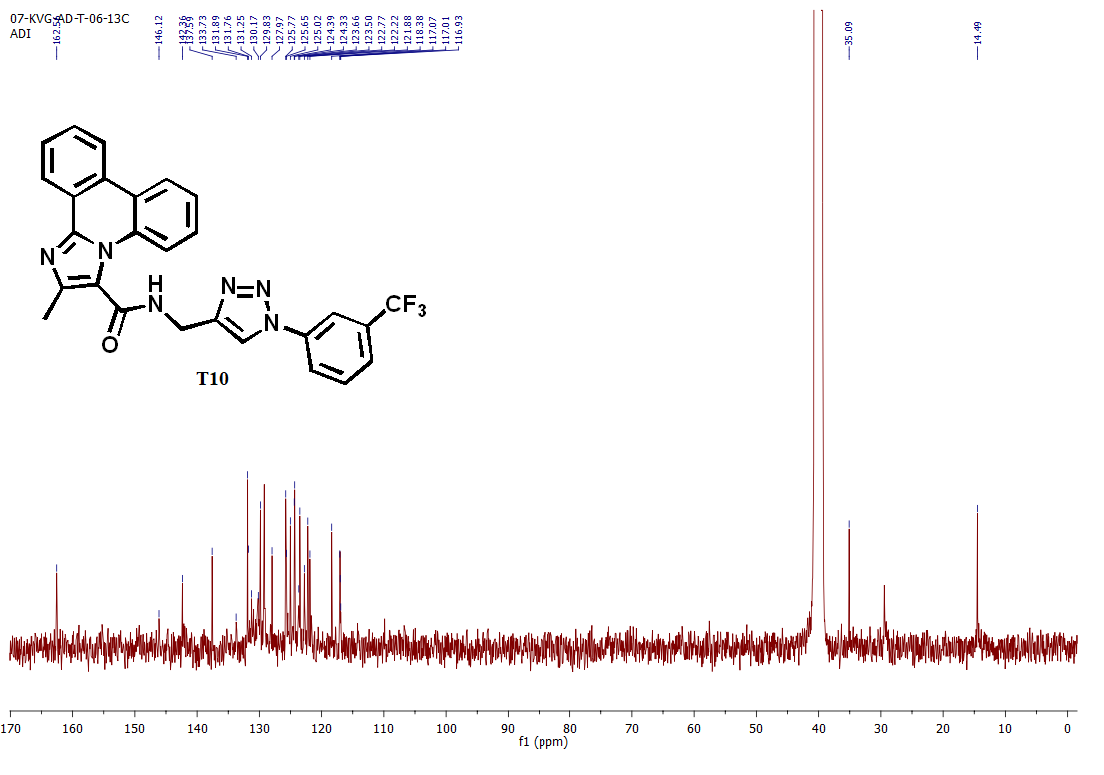
**13CNMR spectrum of compound T06**



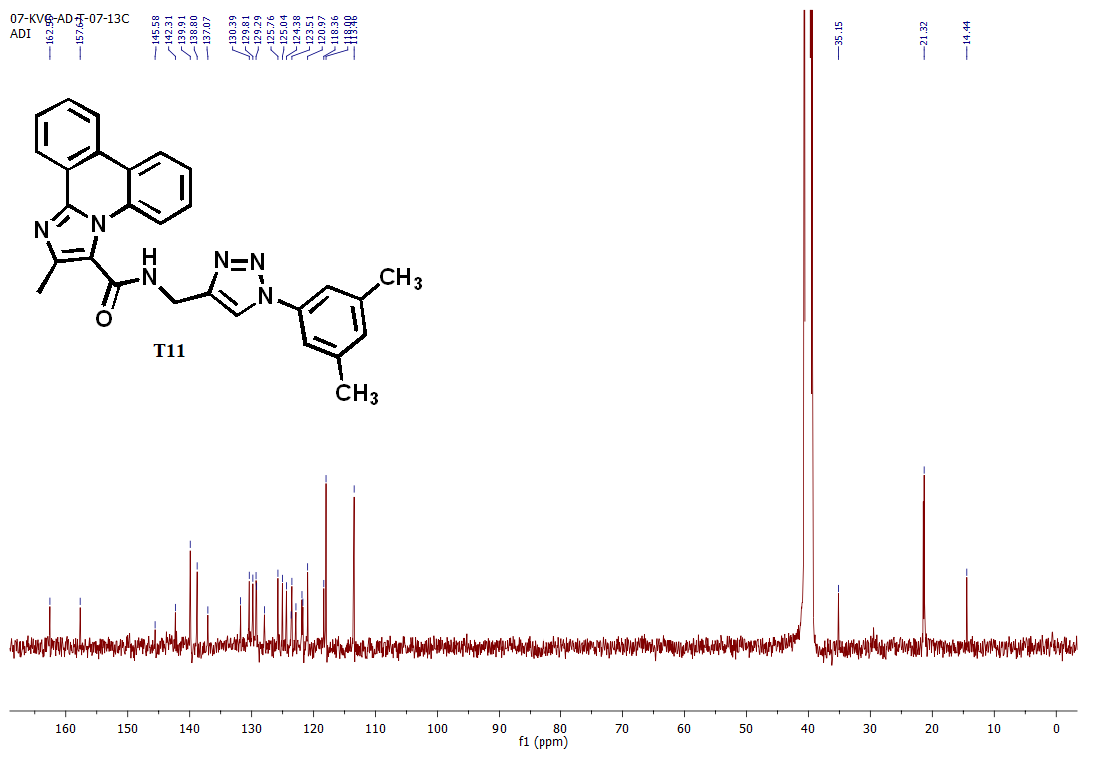
**13CNMR spectrum of compound T07**



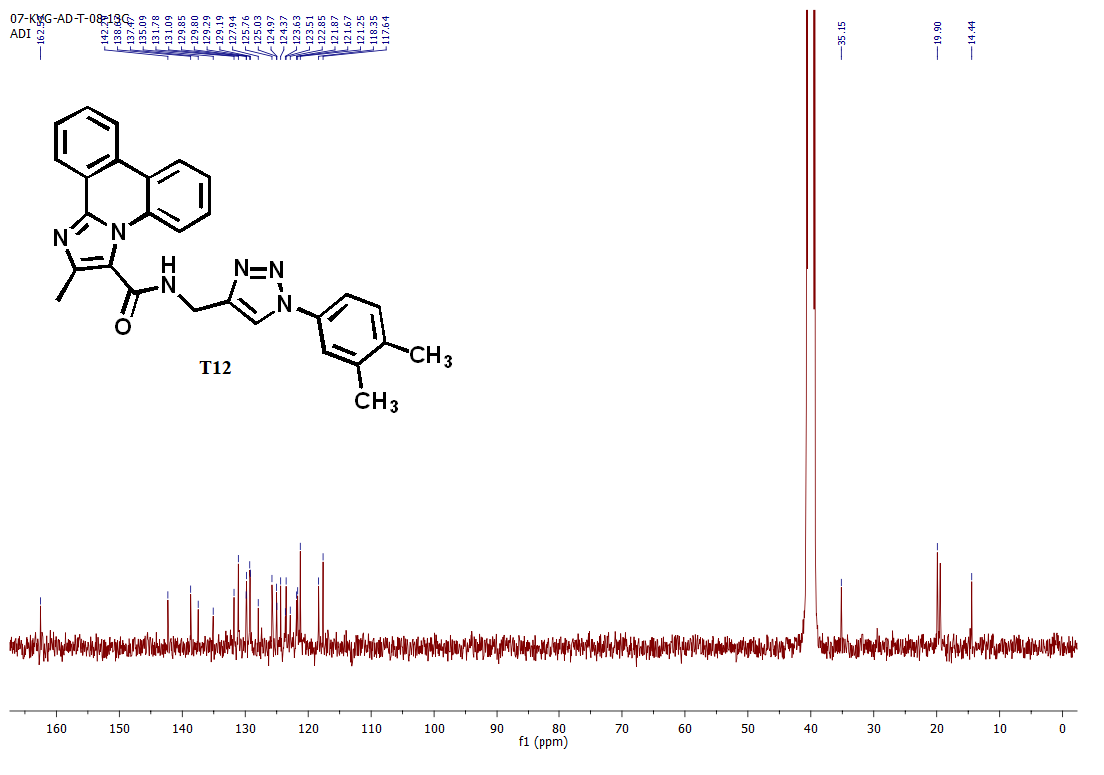
**13CNMR spectrum of compound T09**



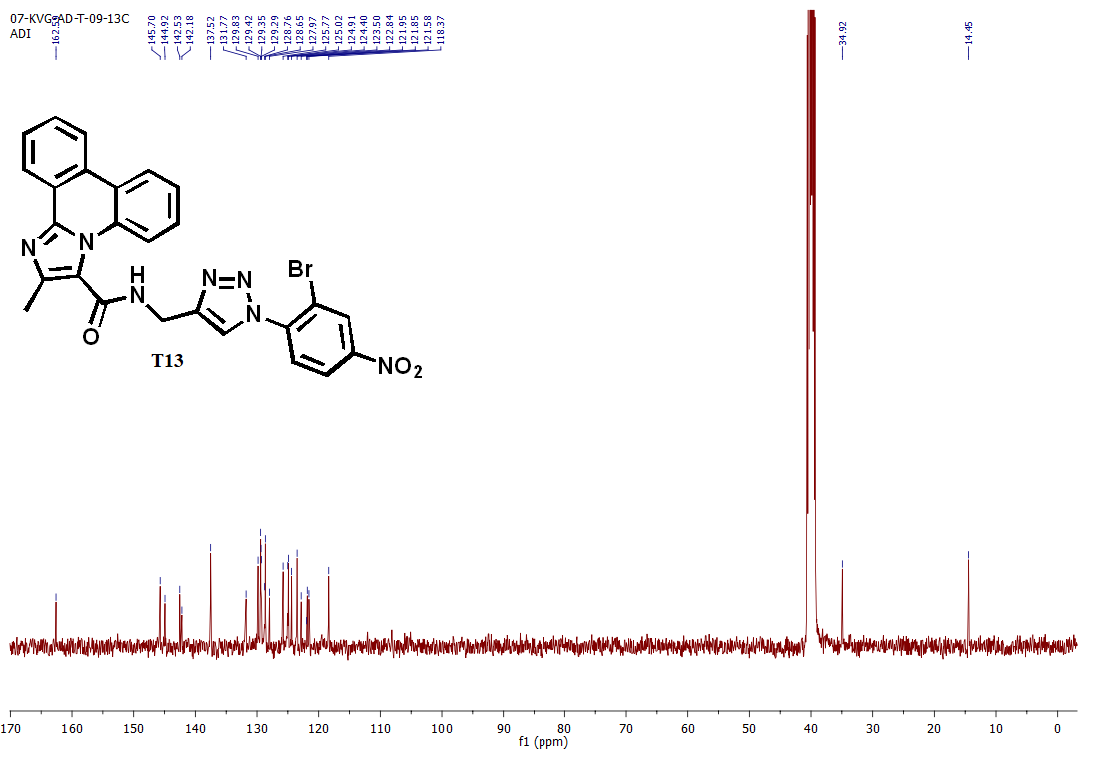
**13CNMR spectrum of compound T10**



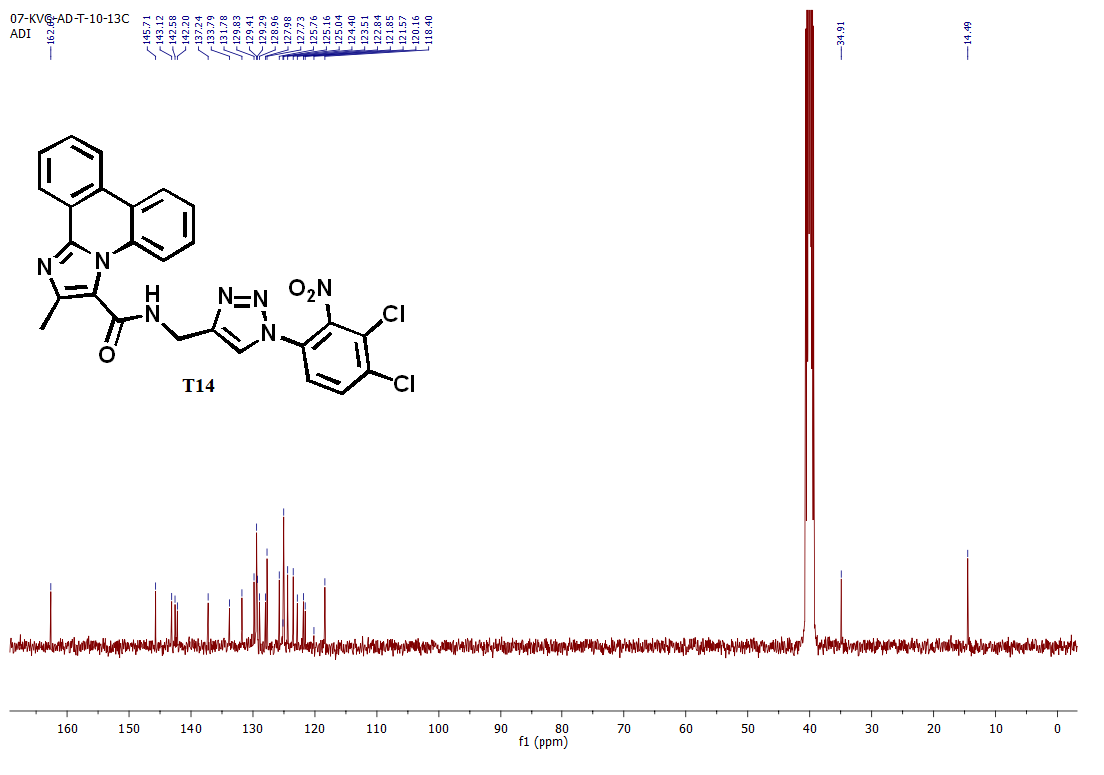
**13CNMR spectrum of compound T11**



**13CNMR spectrum of compound T12**

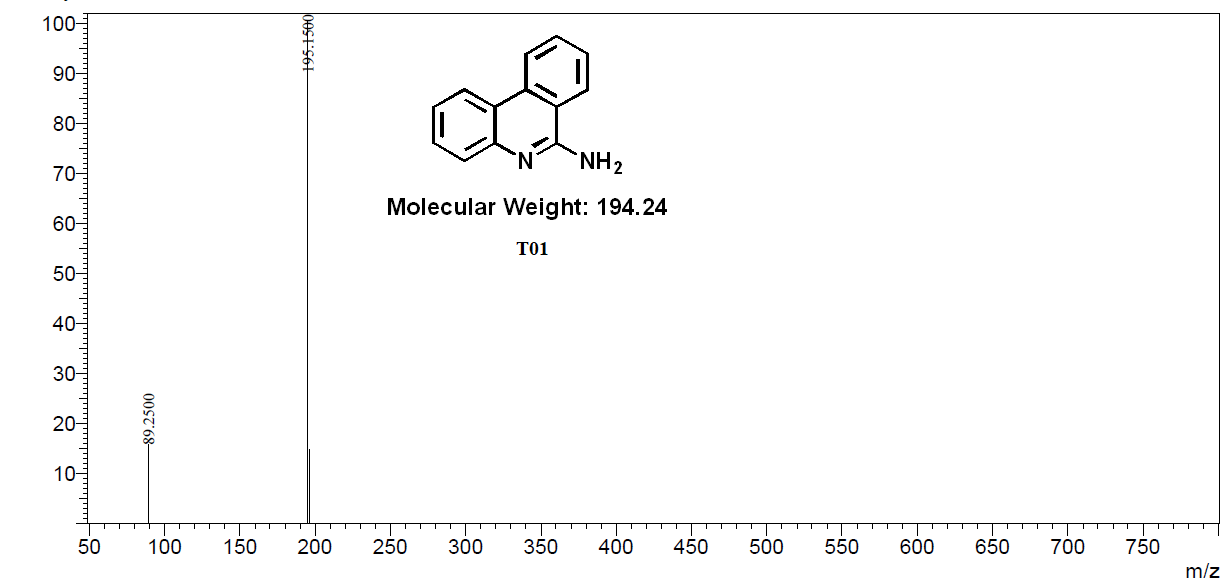


**13CNMR spectrum of compound T13**

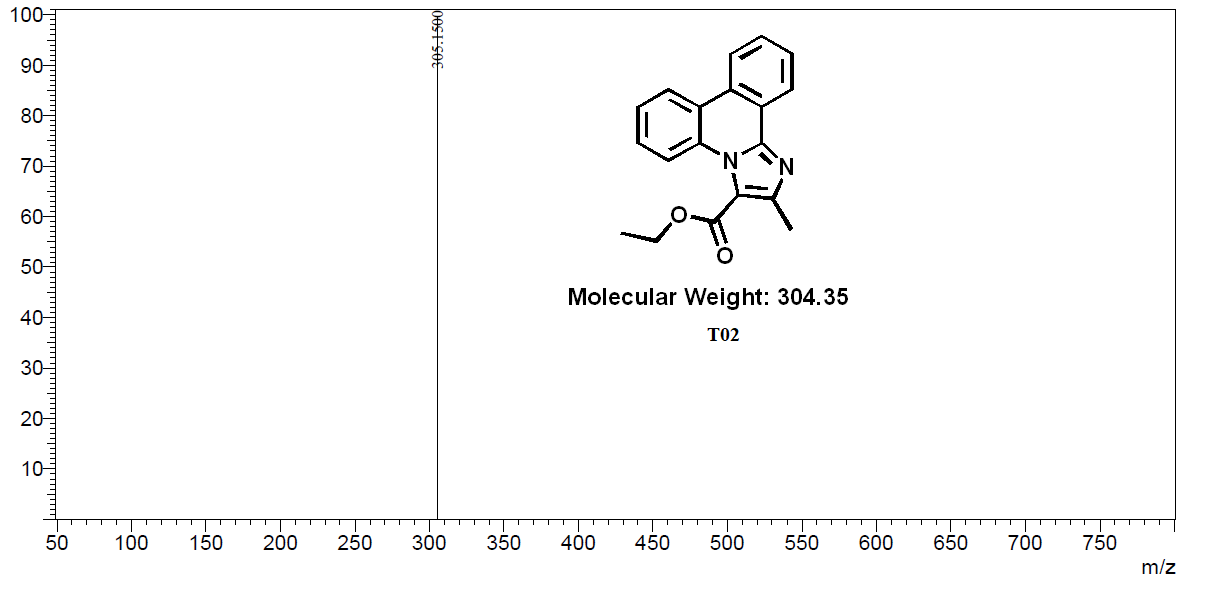


**13CNMR spectrum of compound T14**

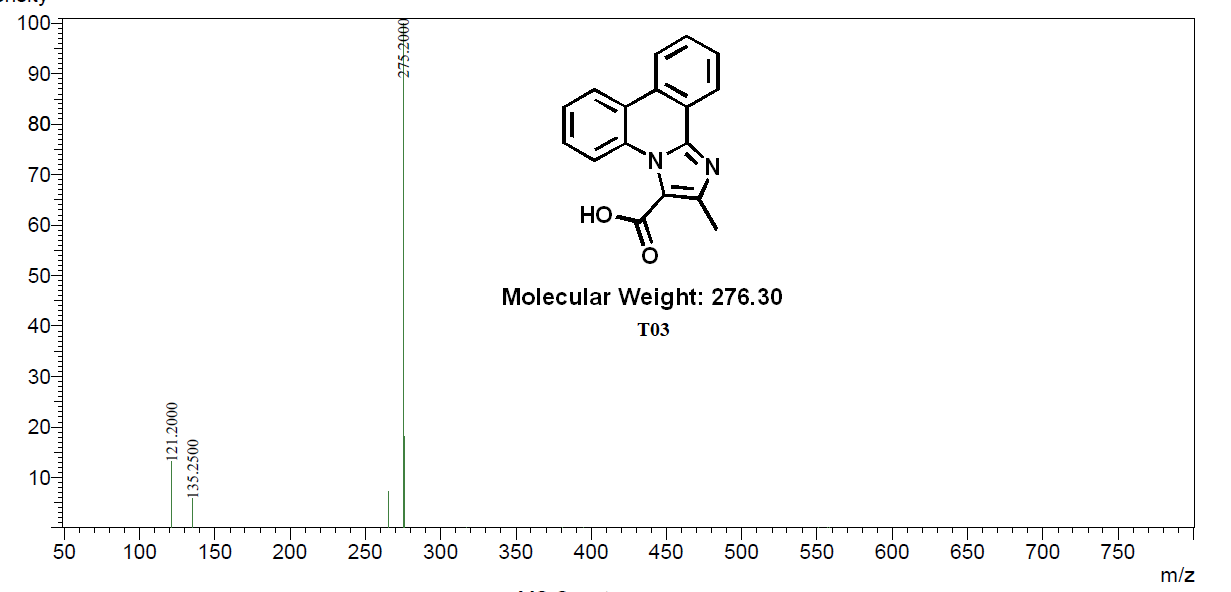
***6. Mass Spectras***

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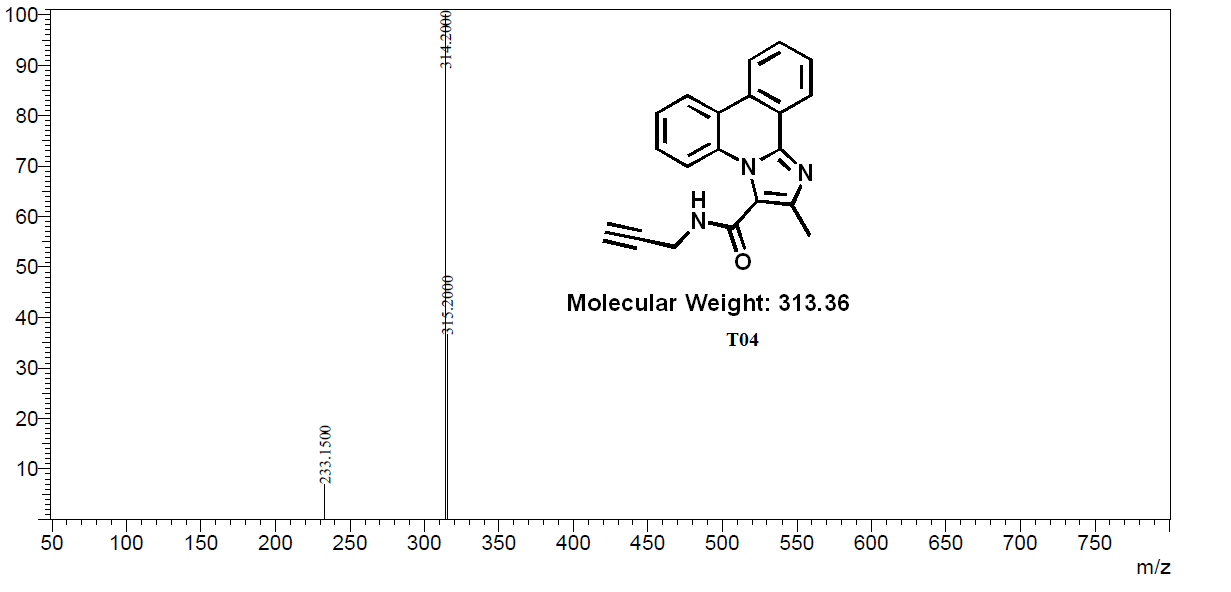
**Mass spectrum of compound T01**

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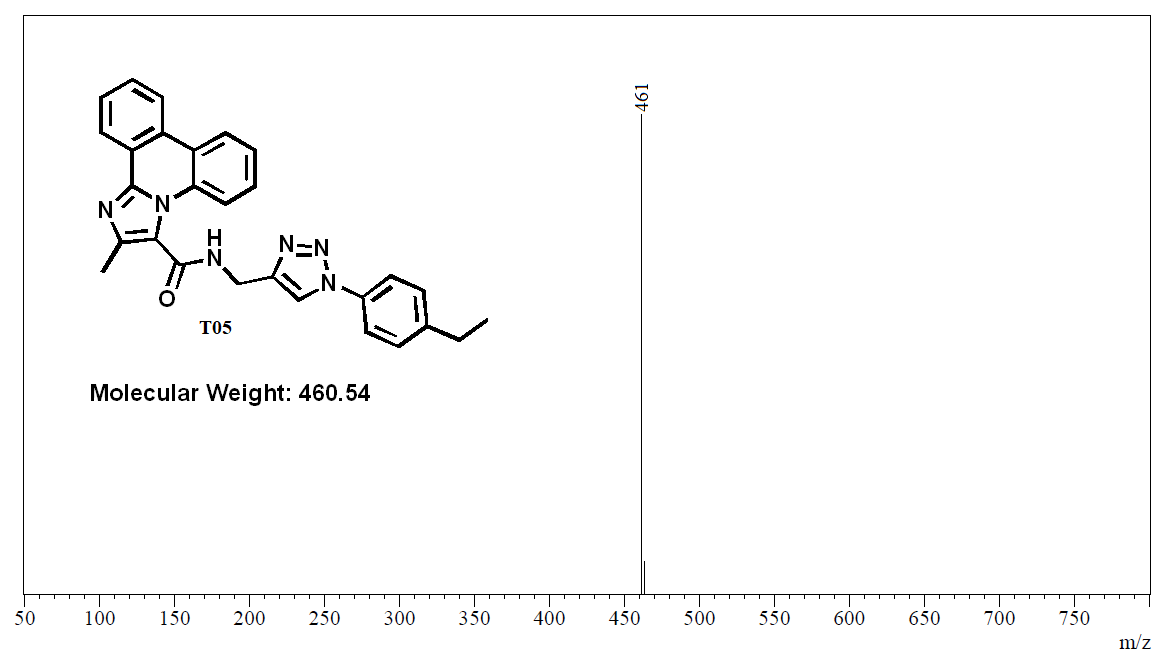
**Mass spectrum of compound T02**

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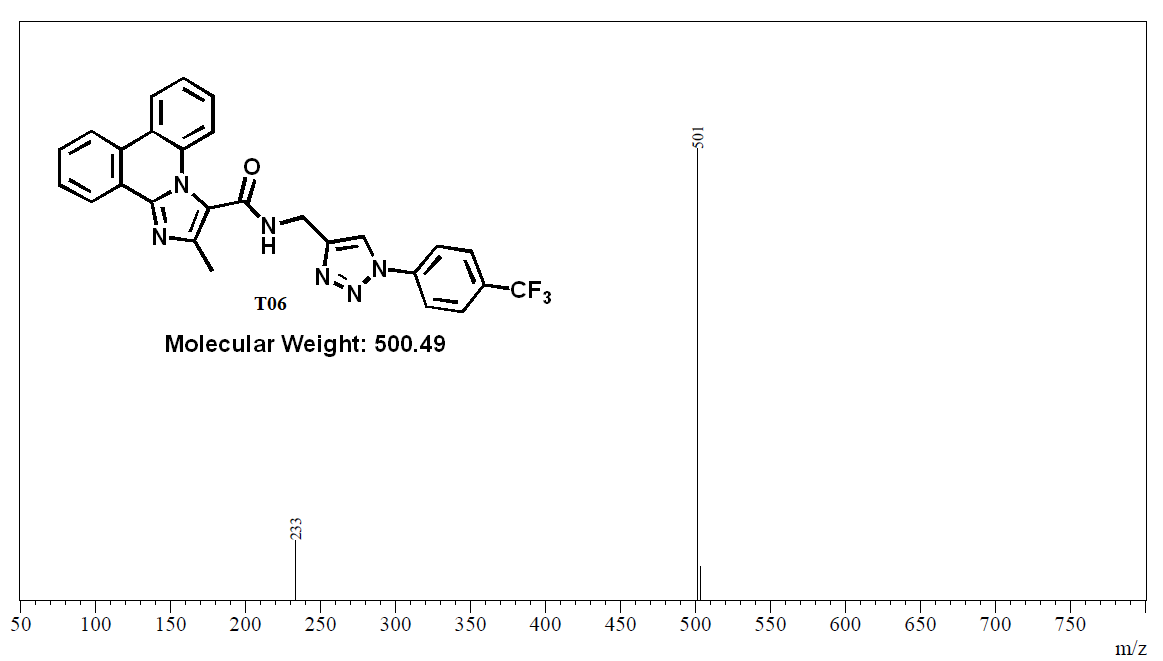
**Mass spectrum of compound T03**

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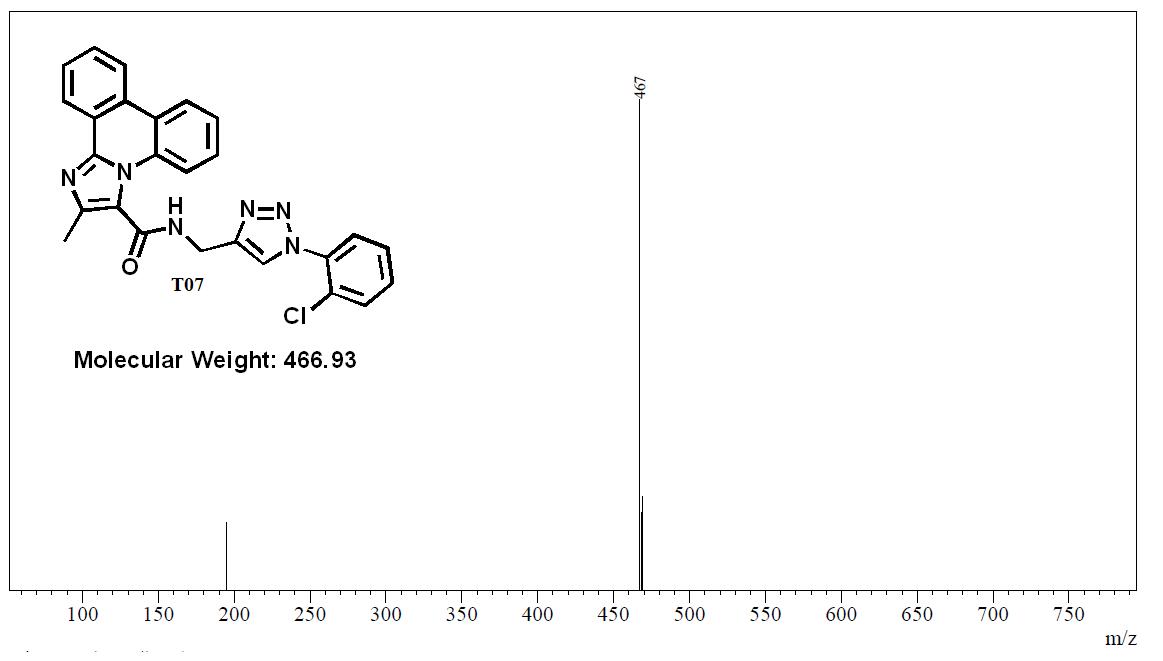
**Mass spectrum of compound T04**



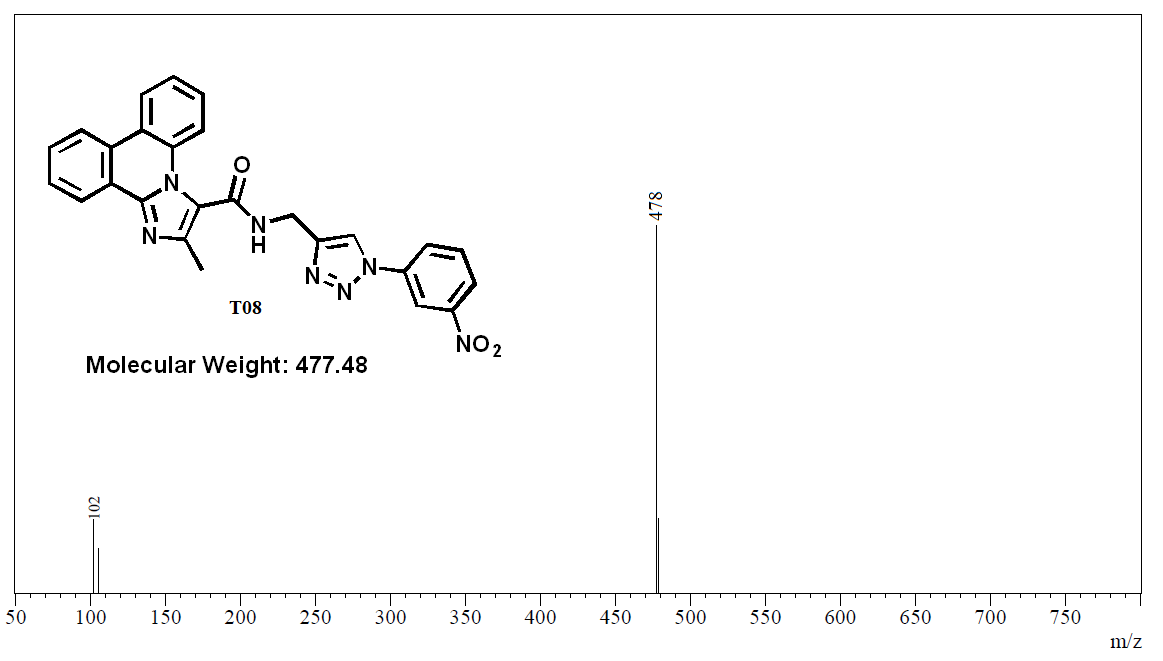
**Mass spectrum of compound T05**



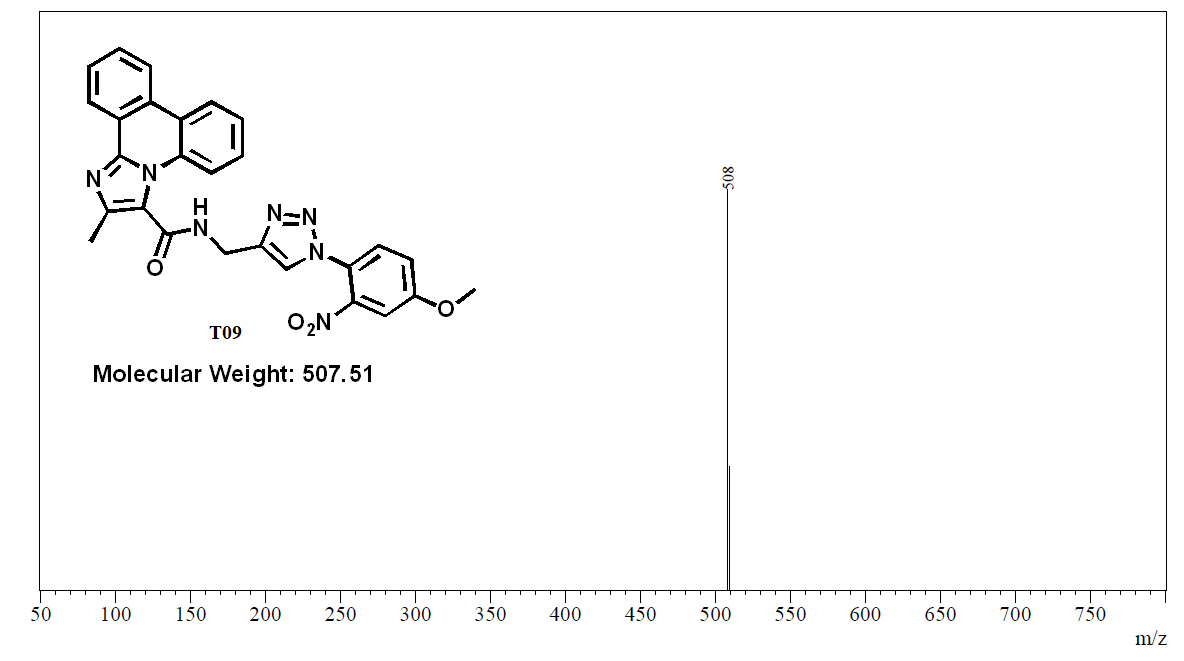
**Mass spectrum of compound T06**



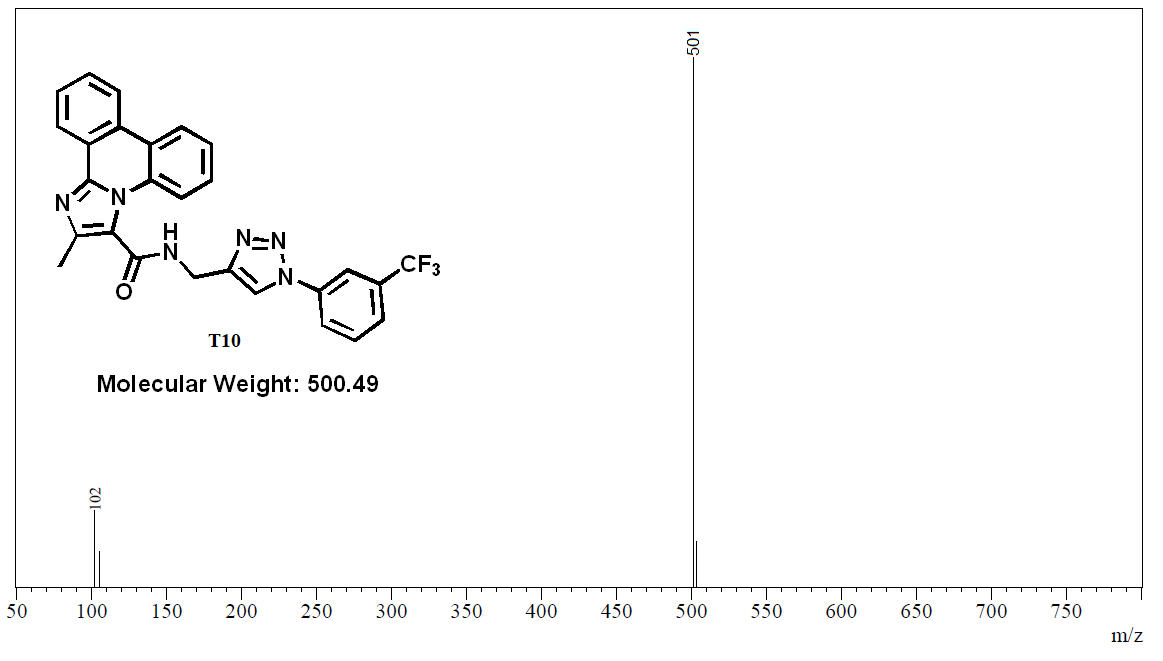
**Mass spectrum of compound T07**



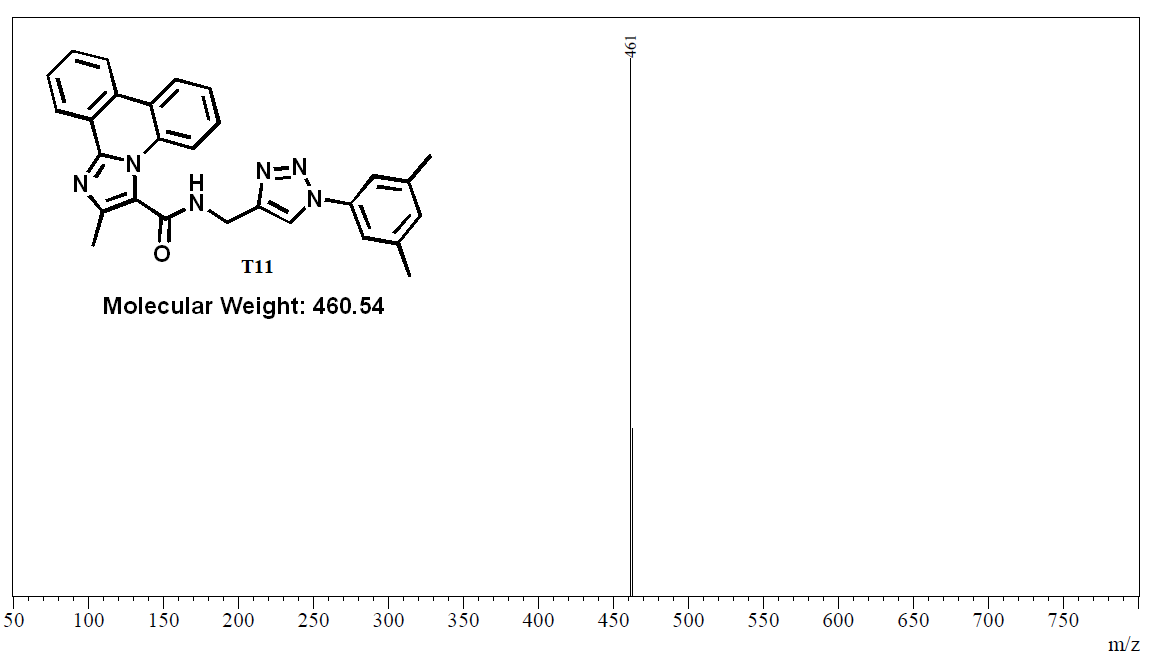
**Mass spectrum of compound T08**



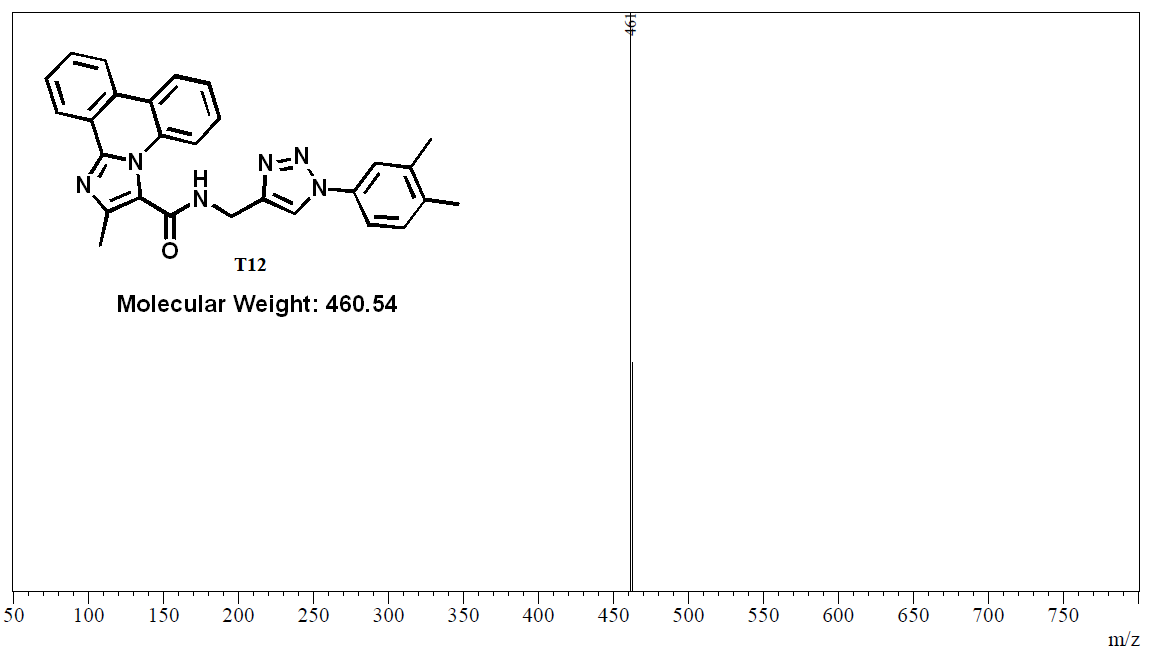
**Mass spectrum of compound T09**



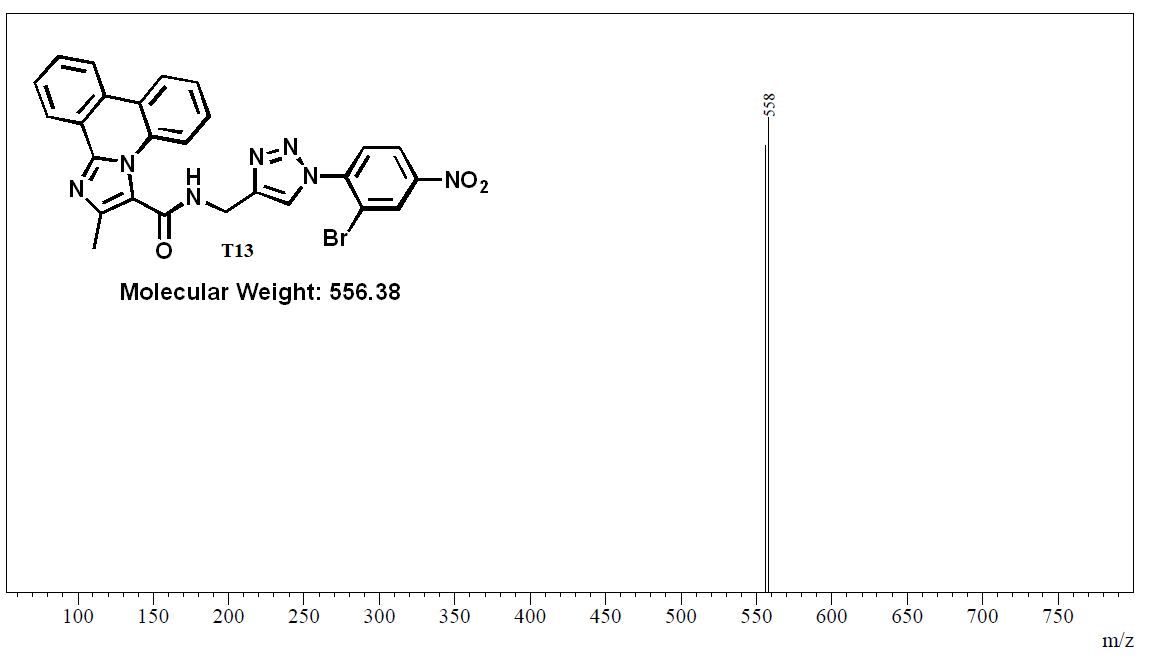
**Mass spectrum of compound T10**



**Mass spectrum of compound T11**



**Mass spectrum of compound T12**



**Mass spectrum of compound T13**



**Mass spectrum of compound T14**

***8. References***

1. De Muylder G, Ang KKH, Chen S, Arkin MR, Engel JC, McKerrow JH. A screen against leishmania intracellular amastigotes: Comparison to a promastigote screen and identification of a host cell-specific hit. *PLoS Negl Trop Dis*. 2011;5(7):e1253. doi:10.1371/journal.pntd.0001253

2. Schrödinger Release 2019-1: QikProp, Schrödinger, LLC, New York, NY, 2019.

3. QikProp Descriptors and Properties PISA, (2015) 2–4.

4. Sander T, Freyss J, Von Korff M, Rufener C. DataWarrior: An open-source program for chemistry aware data visualization and analysis. *J Chem Inf Model*. 2015;55(2):460-473. doi:10.1021/ci500588j

5. Schrödinger Release 2019-1: Maestro, Schrödinger, LLC, New York, NY, 2019.

6. Burley SK, Berman HM, Bhikadiya C, et al. RCSB Protein Data Bank: biological macromolecular structures enabling research and education in fundamental biology, biomedicine, biotechnology and energy. *Nucleic Acids Res*. 2019;47(D1):D464-D474. doi:10.1093/nar/gky1004

7. Schrödinger Release 2019-1: LigPrep, Schrödinger, LLC, New York, NY, 2019.

8. Baiocco P, Colotti G, Franceschini S, Ilari A. Molecular Basis of Antimony Treatment in Leishmaniasis. *J Med Chem*. 2009;52(8):2603-2612. doi:10.1021/jm900185q

9. Schrödinger Release 2019-1: Schrödinger Suite 2019-1 Protein Preparation Wizard; Epik, Schrödinger, LLC, New York, NY, 2019.

10. Friesner RA, Banks JL, Murphy RB, et al. Glide: A New Approach for Rapid, Accurate Docking and Scoring. 1. Method and Assessment of Docking Accuracy. *J Med Chem*. 2004;47(7):1739-1749. doi:10.1021/jm0306430.

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