Combating Oxiinflammaging: Passerini Adducts Tethered with 1,2,3-Triazoles for Enhanced Antioxidant Defense and 5-LOX Inhibition

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1.	**^{1}H and ^{13}C NMR spectra for compounds 4-19**	**S2-S25**
1.1.	**HRMS for compound 19**	**S26**
2.	**Material and Equipment**	**S27**
3.	**Biological Evaluation**	**S27**
3.1.	**Evaluating the antioxidant activity**	**S27**
3.1.1.	**Thiobarbituric Acid Reactive Substances (TBARS) assay**	**S27**
3.1.2.	**Nitric Oxide (NO) scavenging assay**	**S27**
3.1.3.	**Free radical scavenging activity by DPPH• (2, 2-diphenyl-1-picrylhydrazyl radical)**	**S28**
3.2.	**Evaluating the anti-inflammatory potential**	**S28**
3.2.1.	***In vitro* 5-Lipoxygenase (5-LOX) inhibitory assay**	**S28**
3.2.2.	**Membrane stabilization (RBC's) assay**	**S28**
4.	**Conformational analysis study**	**S29**
5.	**Molecular docking study**	**S30**
6.	**Data analysis and statistics**	**S30**
7.	**References**	**S31**

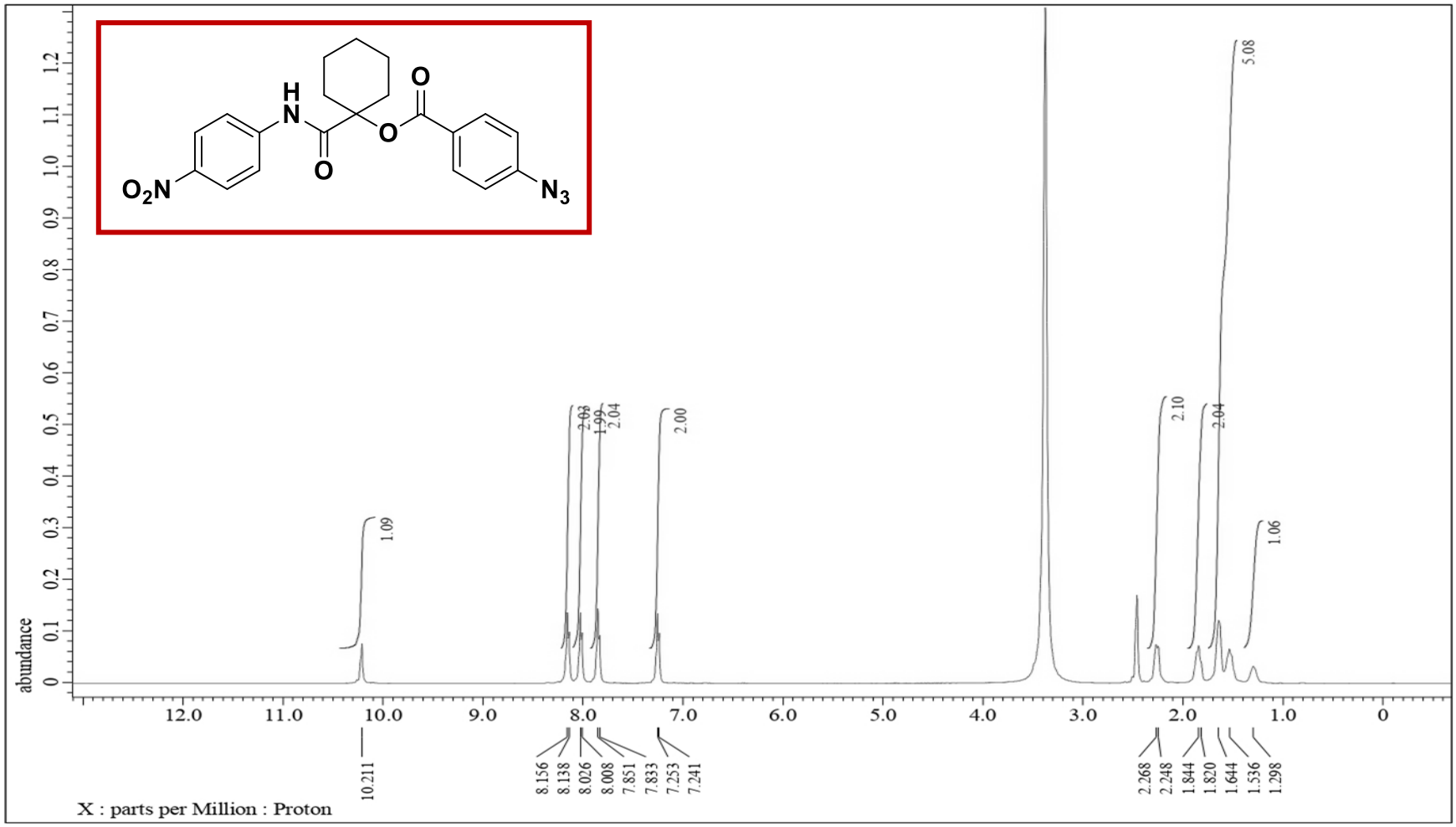


Figure S1. ^{1}H-NMR (500 MHz, DMSO-d_6) spectrum of **4**

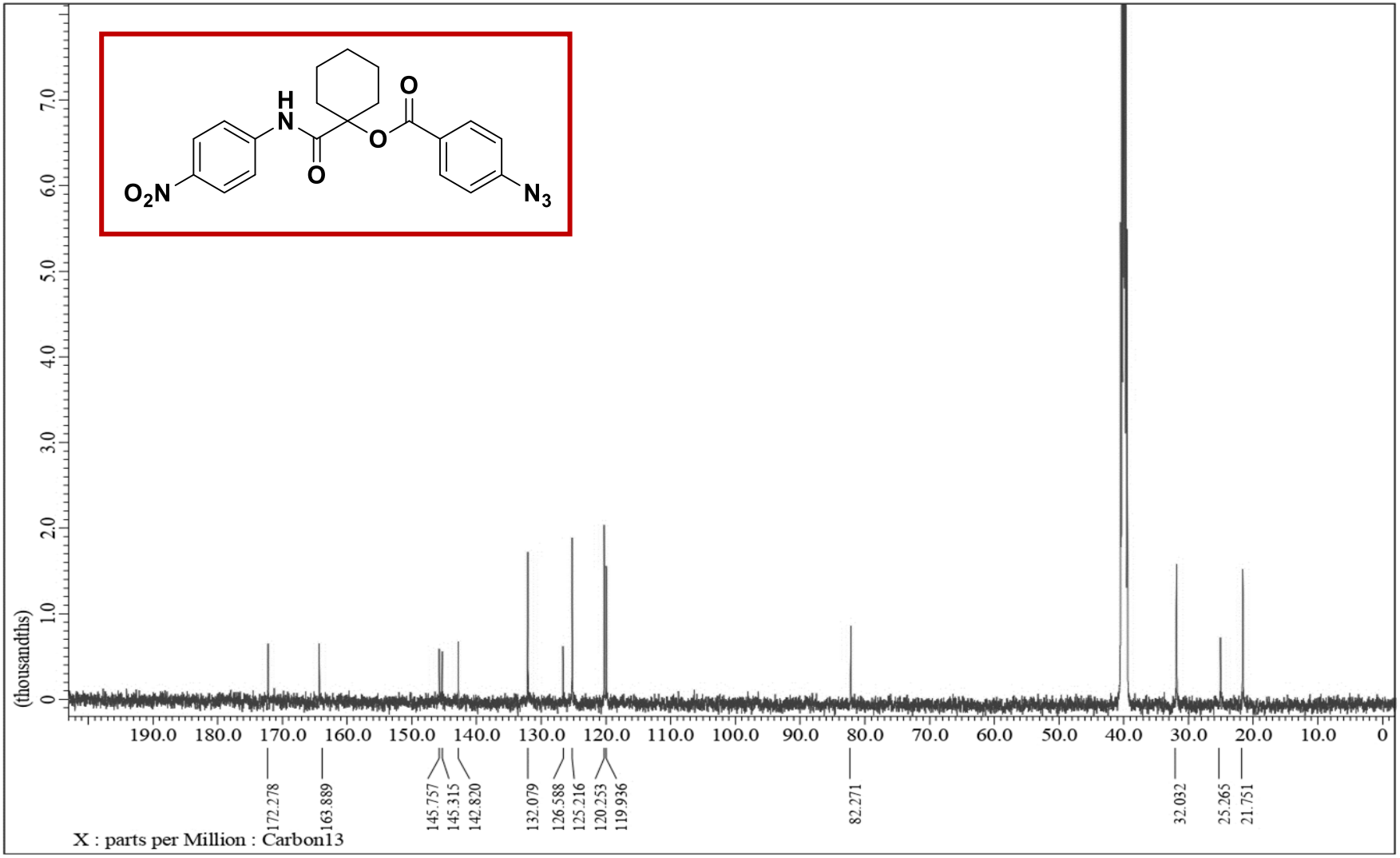


Figure S2. ^{13}C-NMR (125 MHz, DMSO-d_6) spectrum of **4**

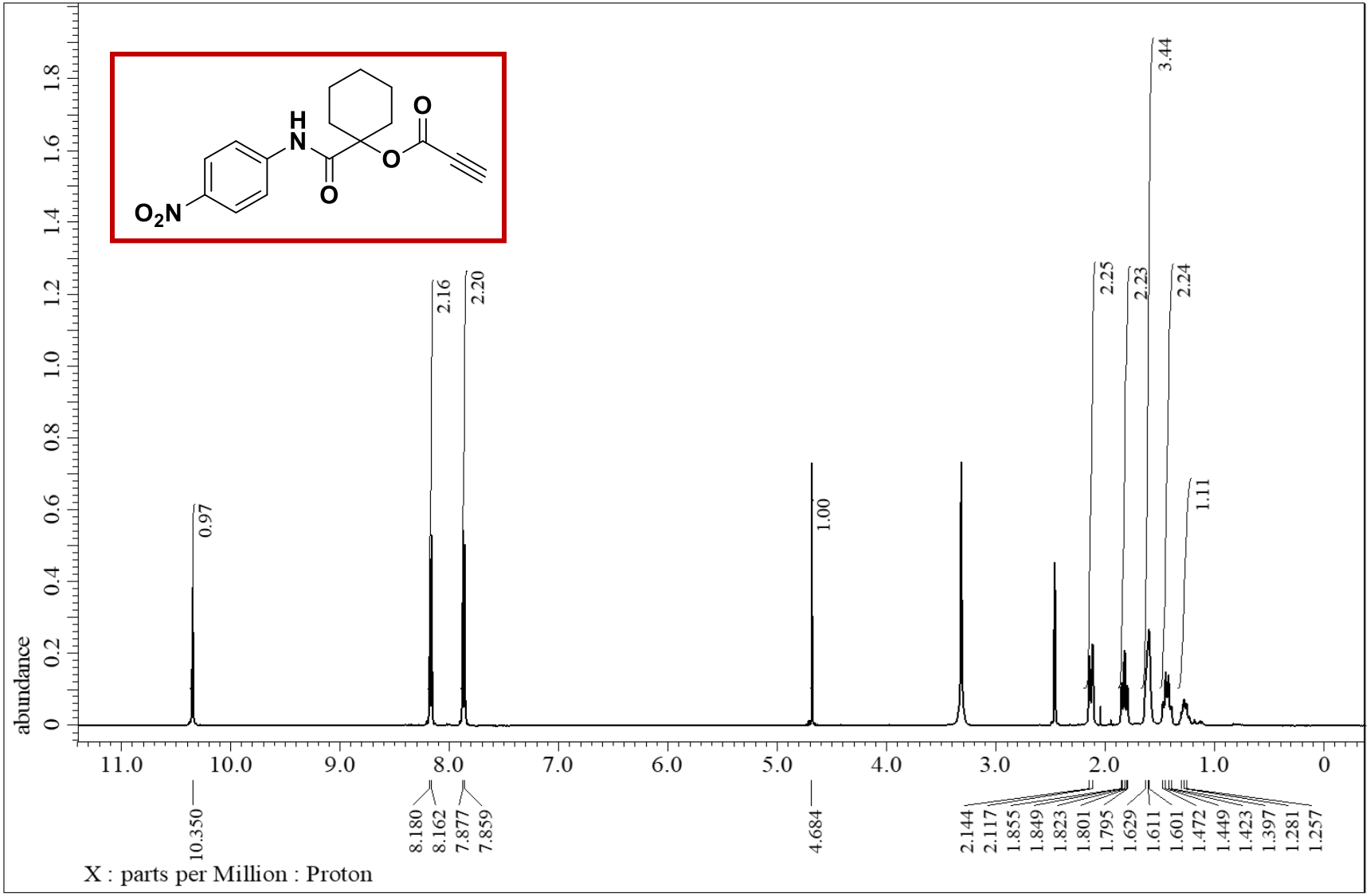


Figure S3. ^{1}H-NMR (500 MHz, DMSO-d_6) spectrum of **5**

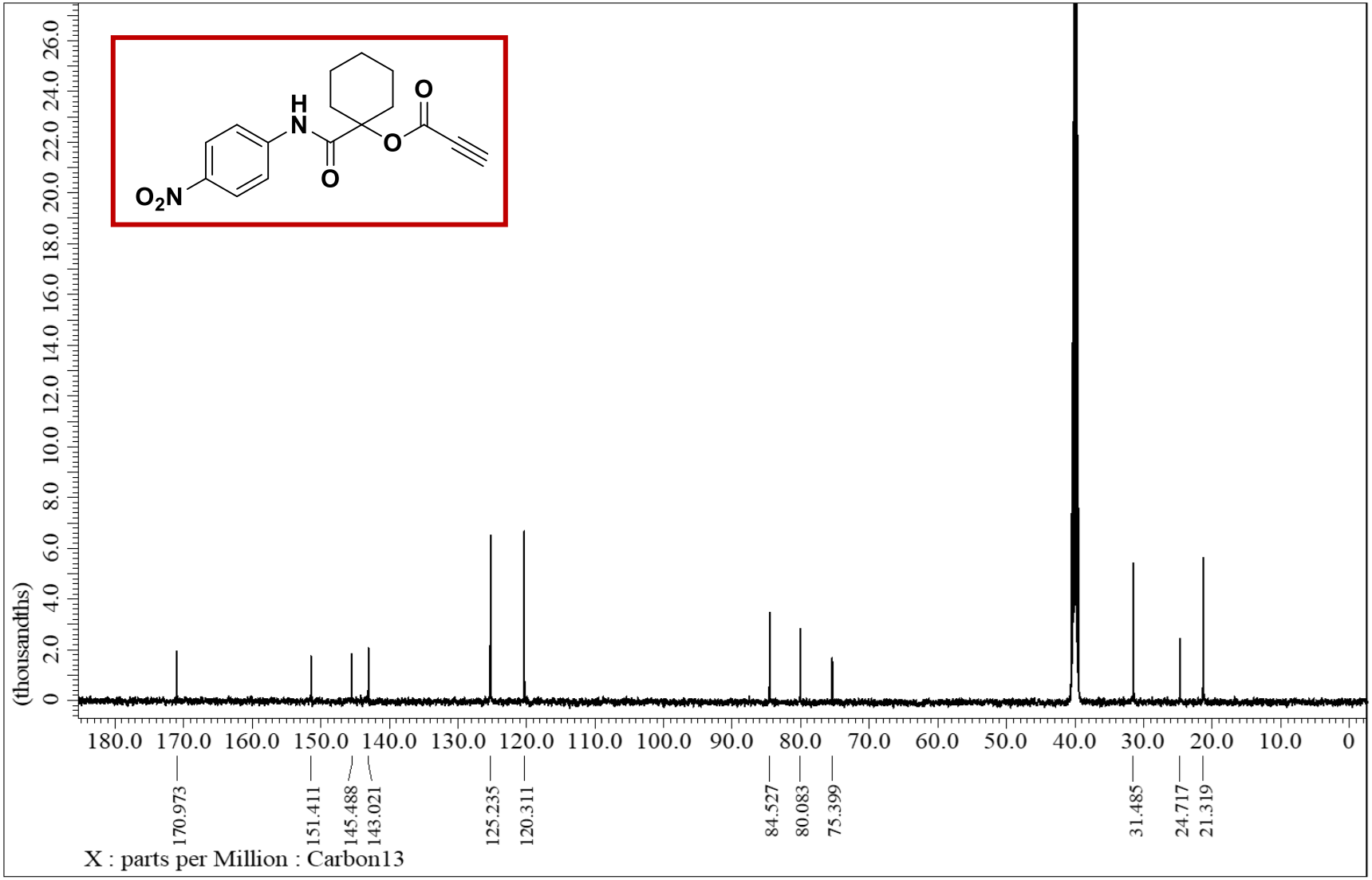


Figure S4. ^{13}C-NMR (125 MHz, DMSO-d_6) spectrum of **5**

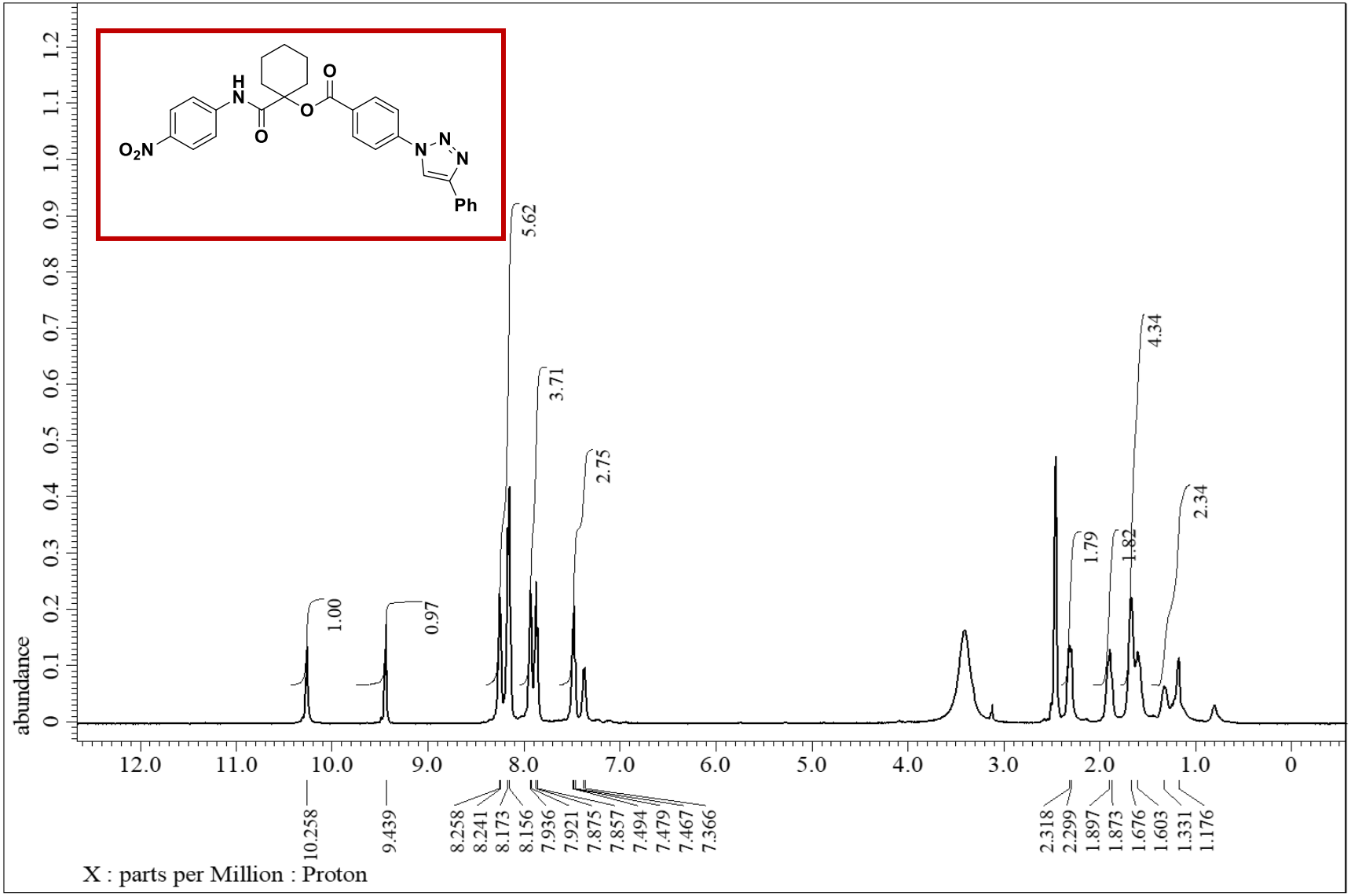


Figure S5. ^{1}H-NMR (500 MHz, DMSO-d_6) spectrum of **7**

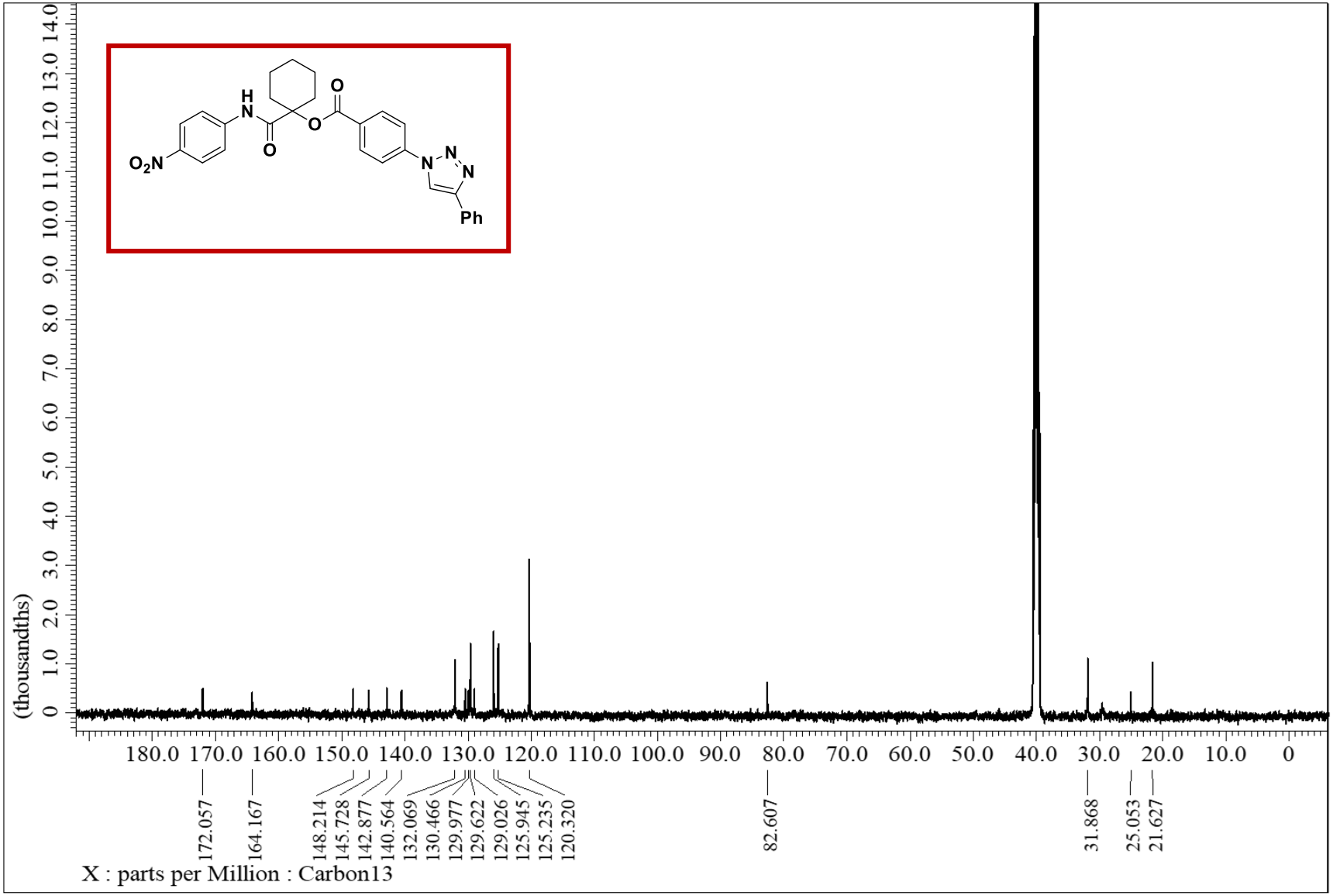


Figure S6. ^{13}C-NMR (125 MHz, DMSO-d_6) spectrum of **7**

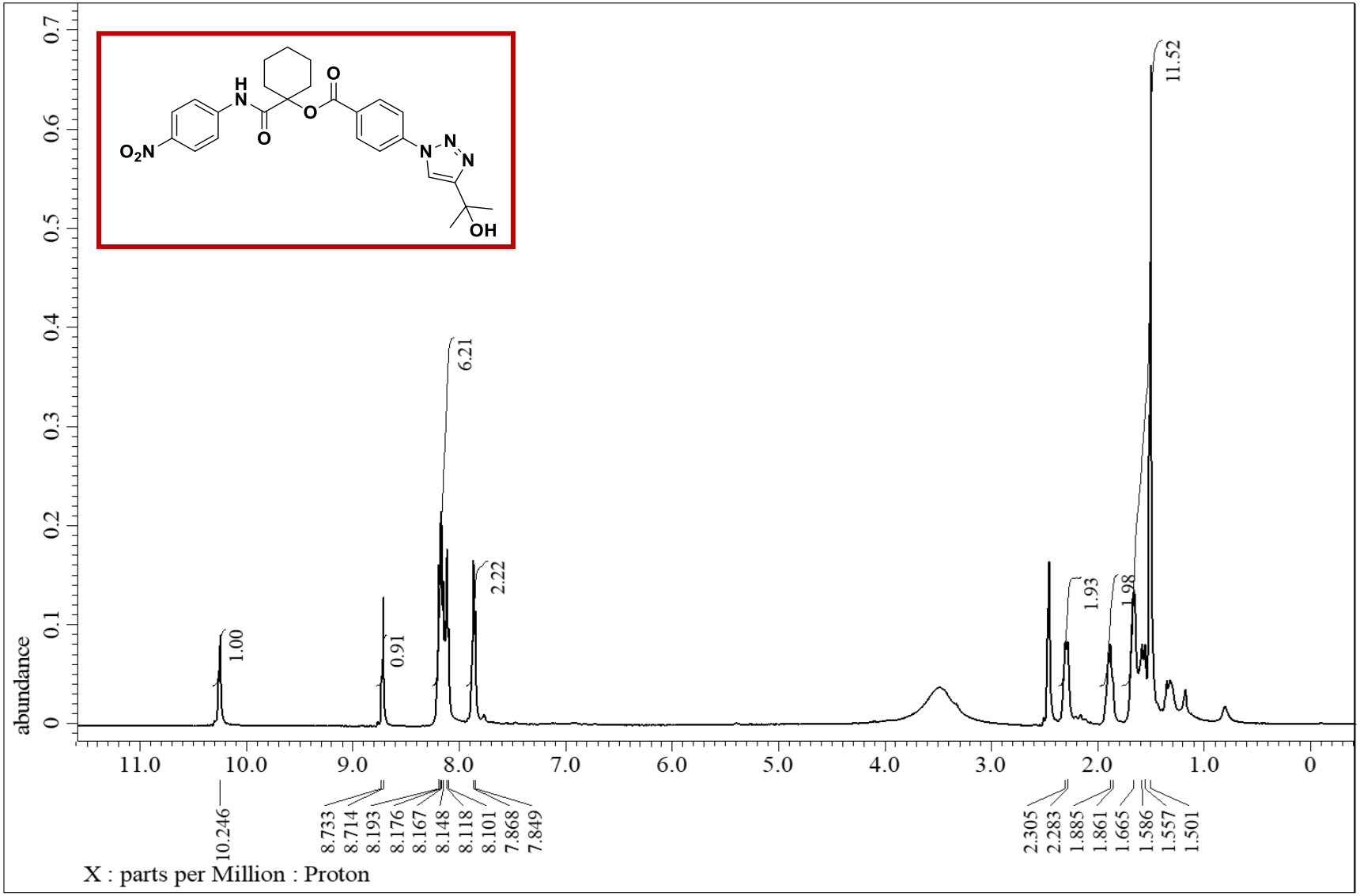


Figure S7. ^{1}H-NMR (500 MHz, DMSO-d_6) spectrum of **8**

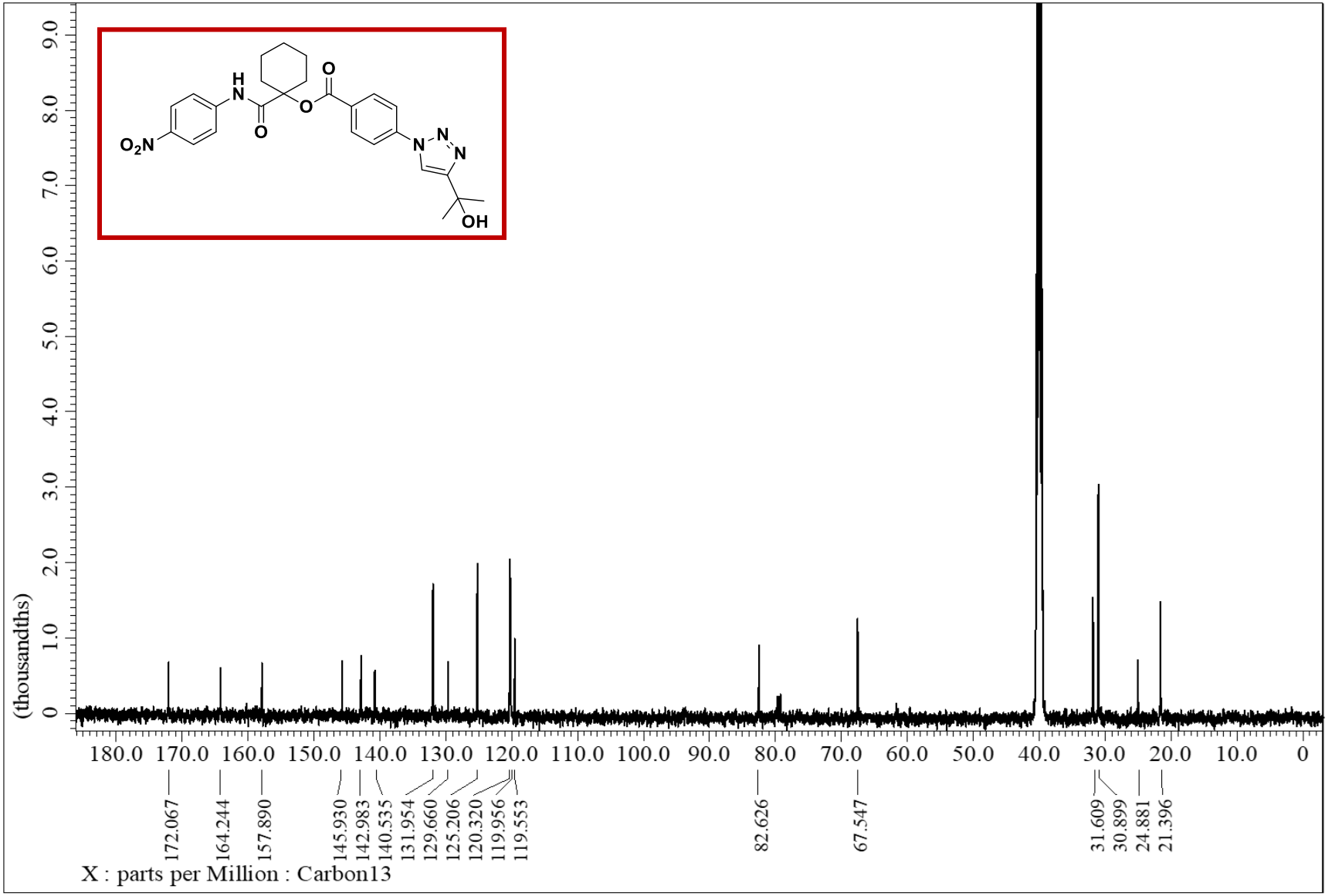


Figure S8. ^{13}C-NMR (125 MHz, DMSO-d_6) spectrum of **8**

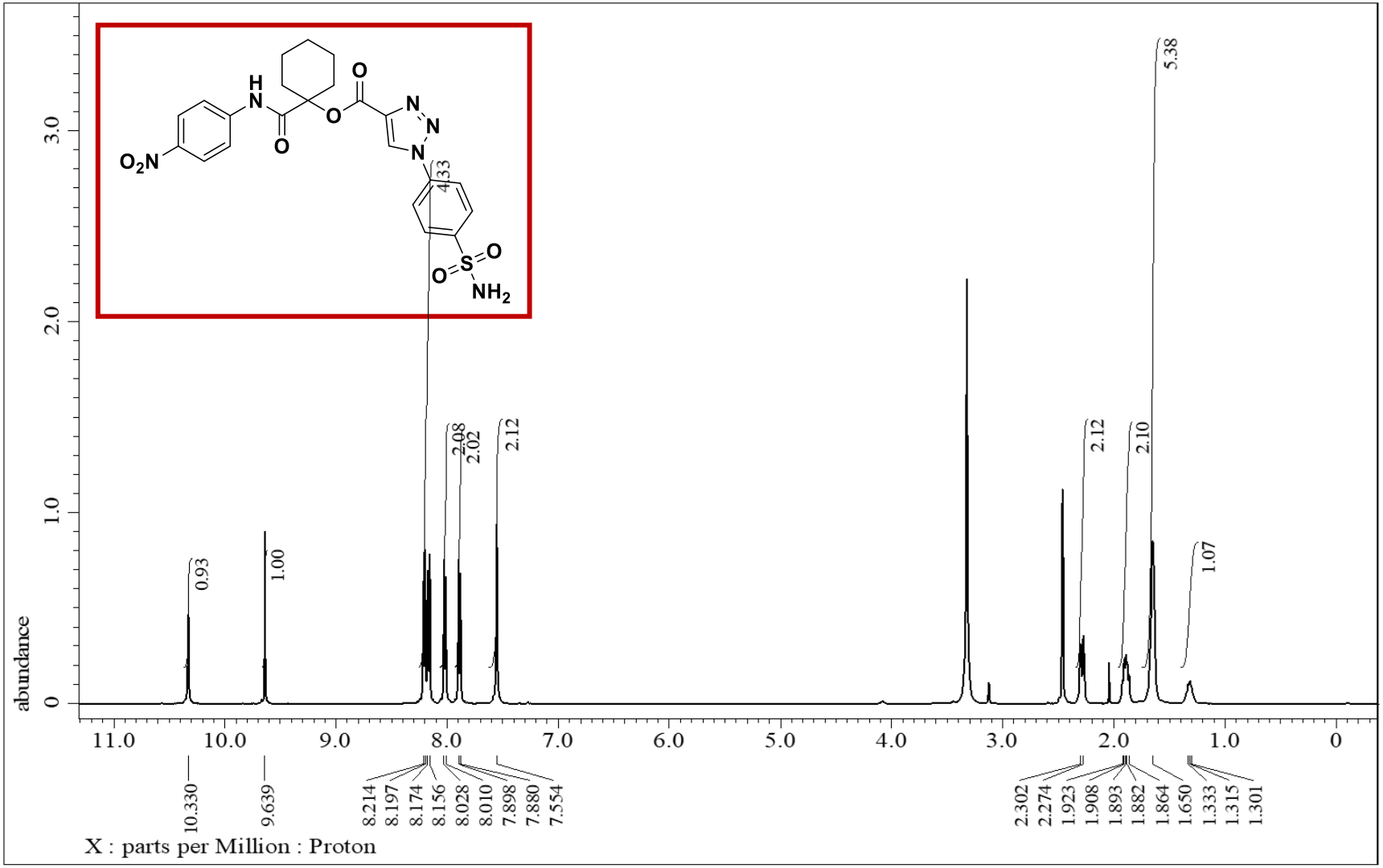


Figure S9. ^{1}H-NMR (500 MHz, DMSO-d_6) spectrum of **11**

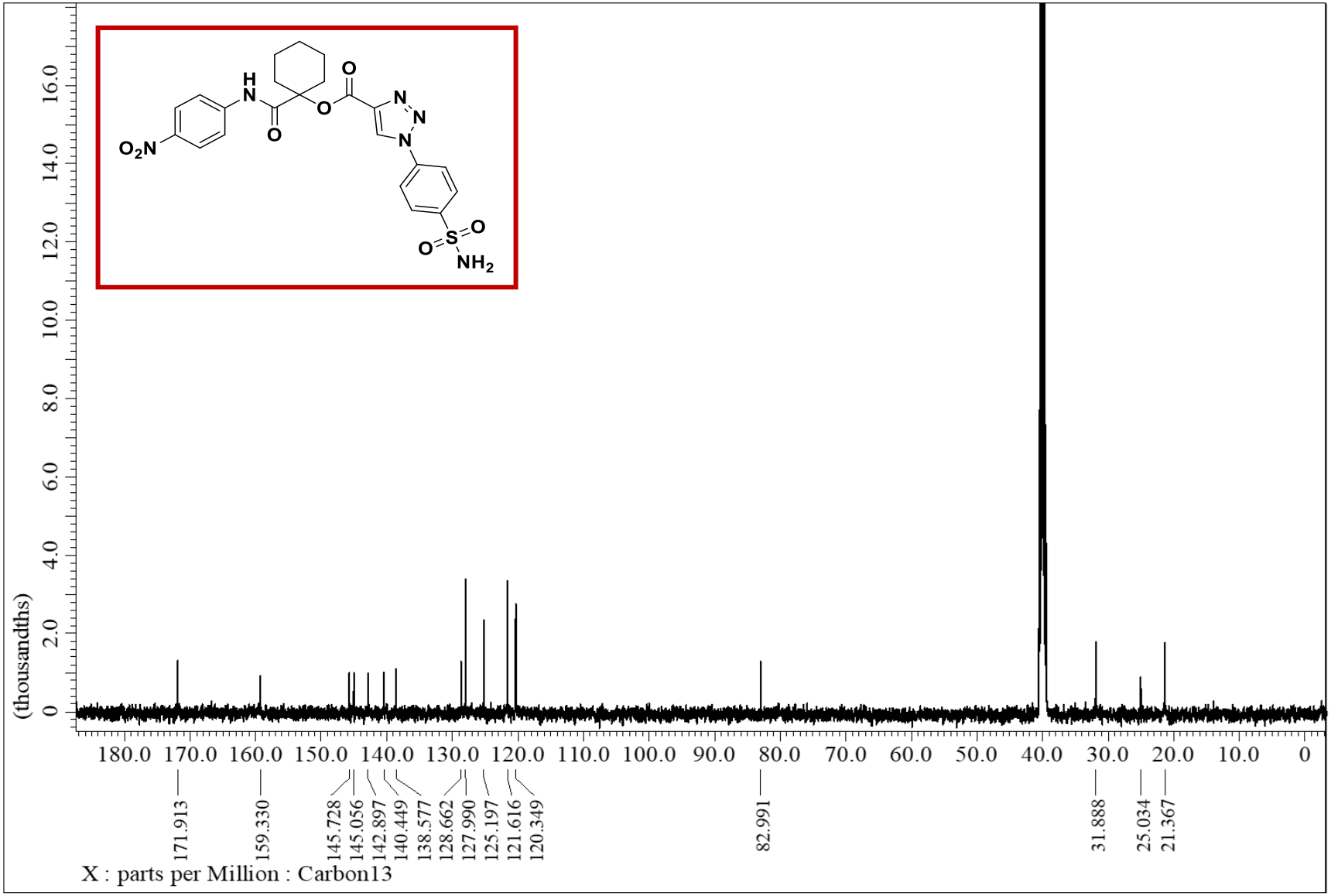


Figure S10. ^{13}C-NMR (500 MHz, DMSO-d_6) spectrum of **11**

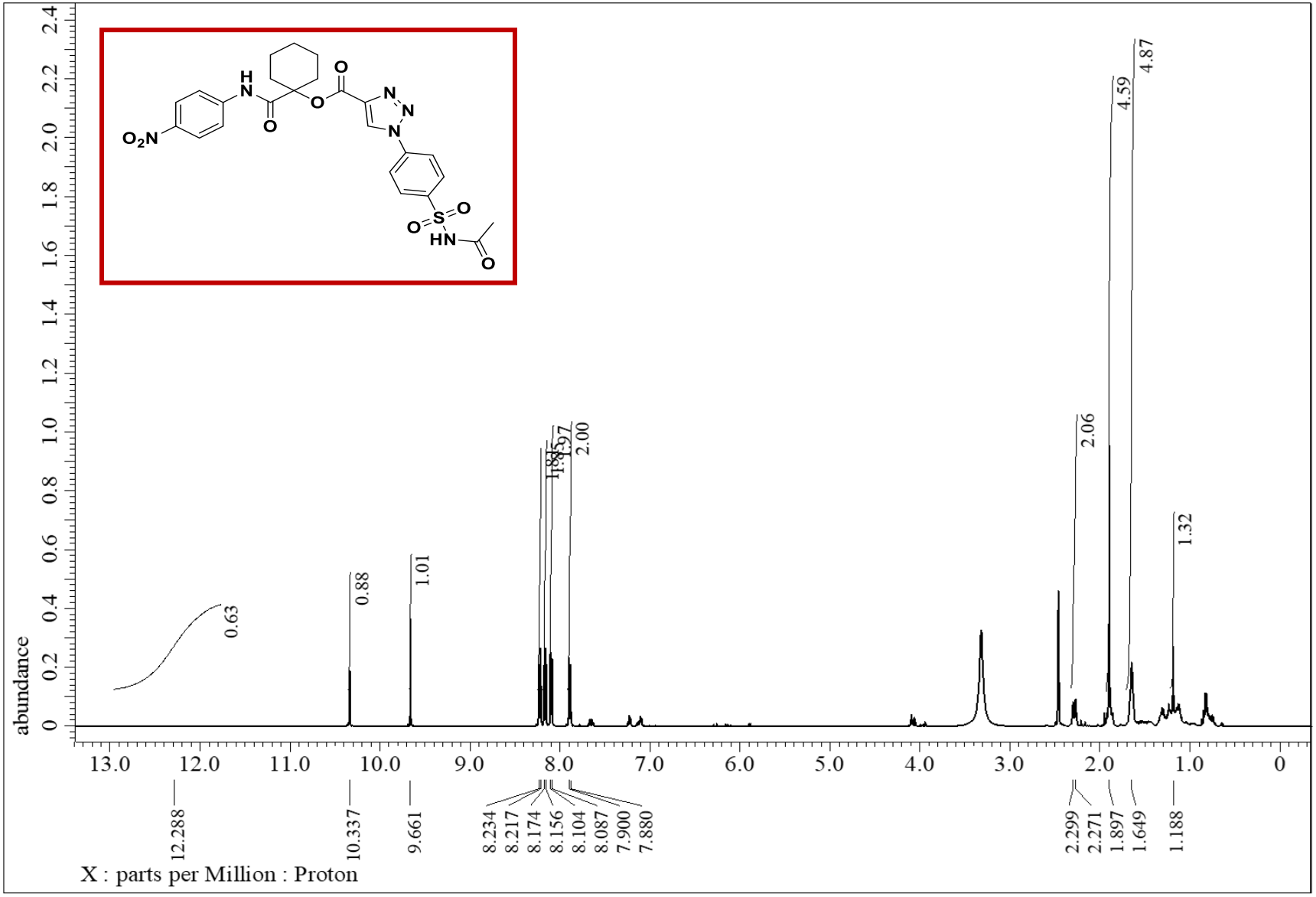


Figure S11. ^{1}H-NMR (500 MHz, DMSO-d_6) spectrum of **12**

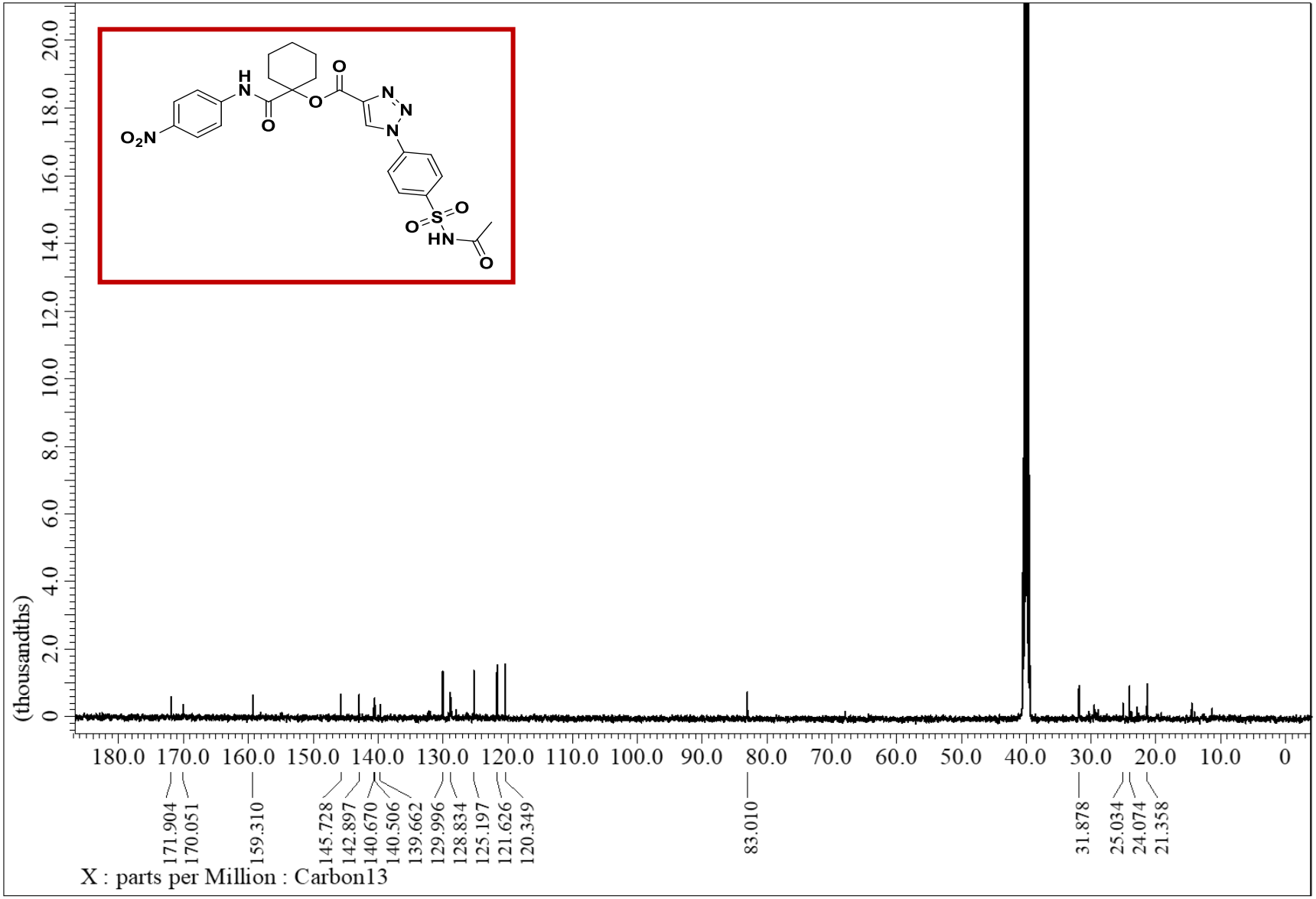


Figure S12. ^{13}C-NMR (125 MHz, DMSO-d_6) spectrum of **12**

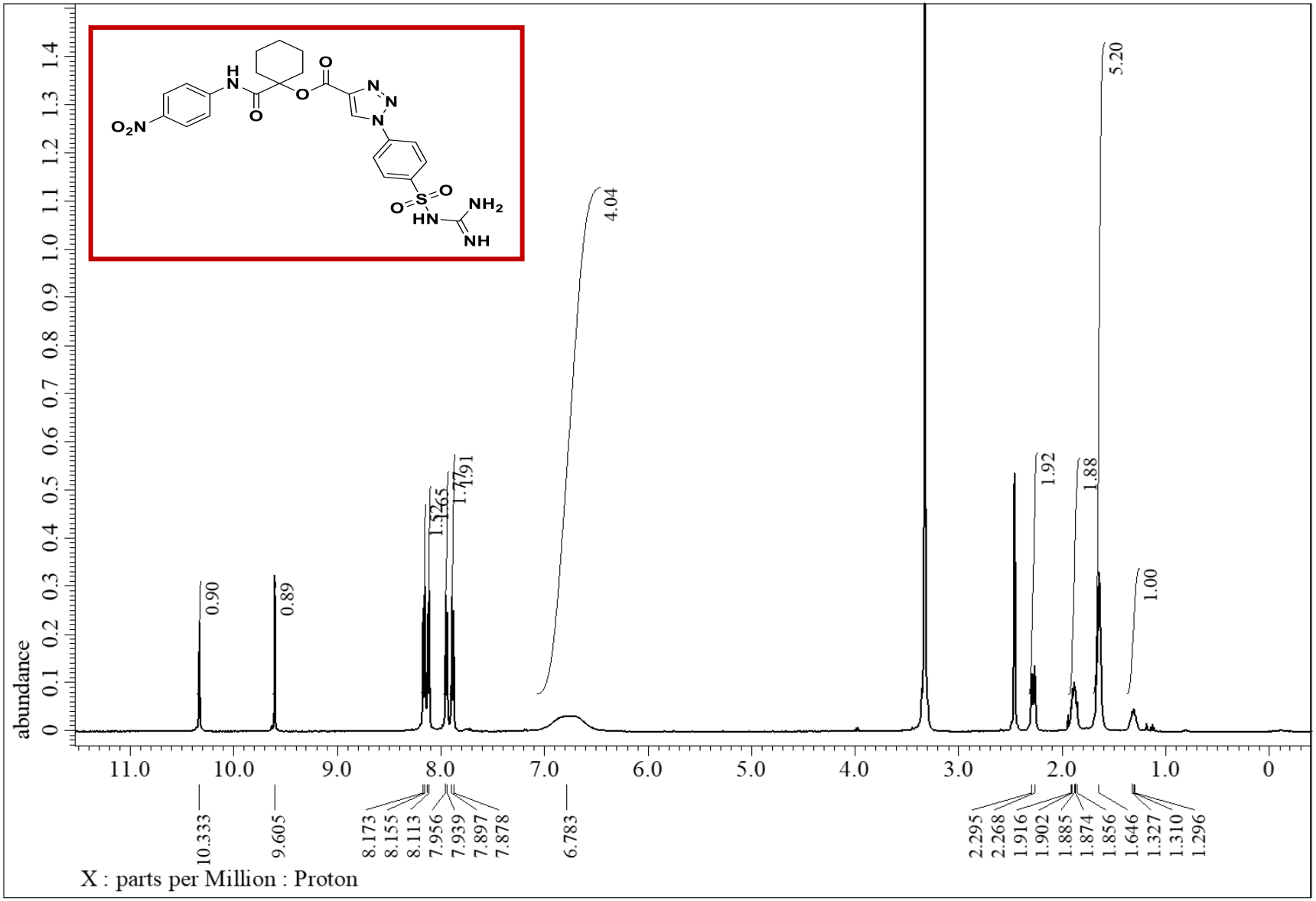


Figure S13. ^{1}H-NMR (500 MHz, DMSO-d_6) spectrum of **13**

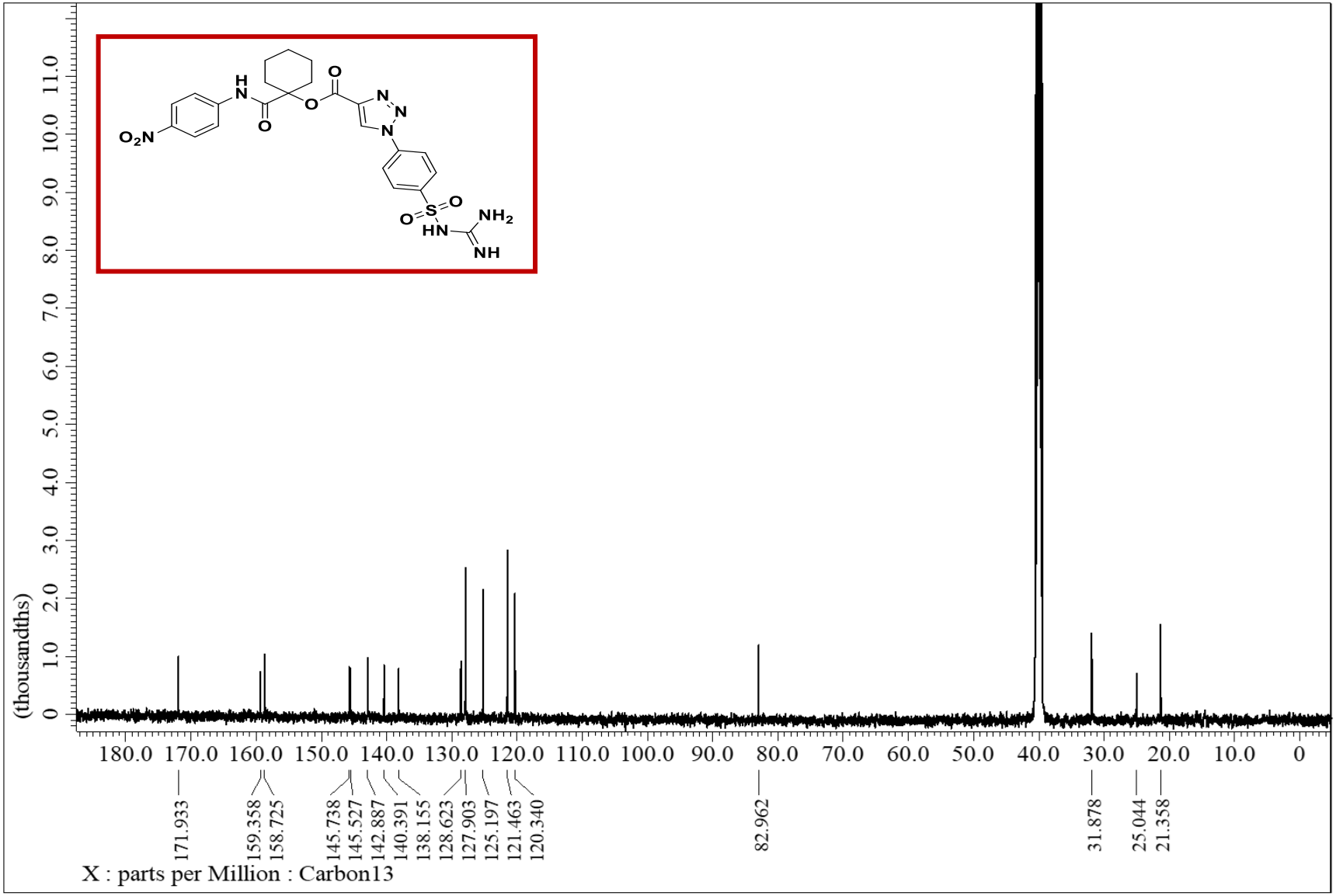


Figure S14. ^{13}C-NMR (125 MHz, DMSO-d_6) spectrum of **13**

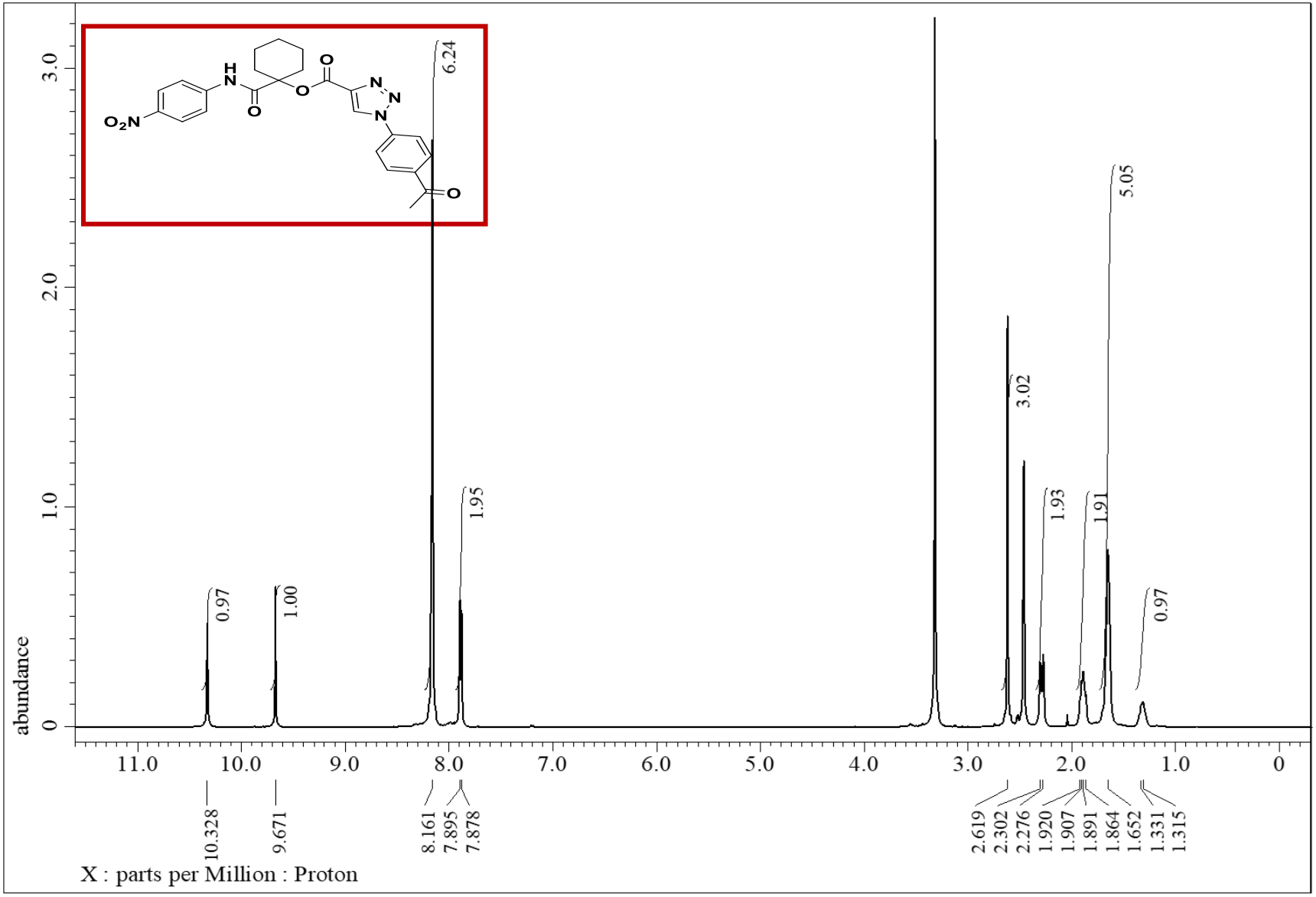


Figure S15. ^{1}H-NMR (500 MHz, DMSO-d_6) spectrum of **14**

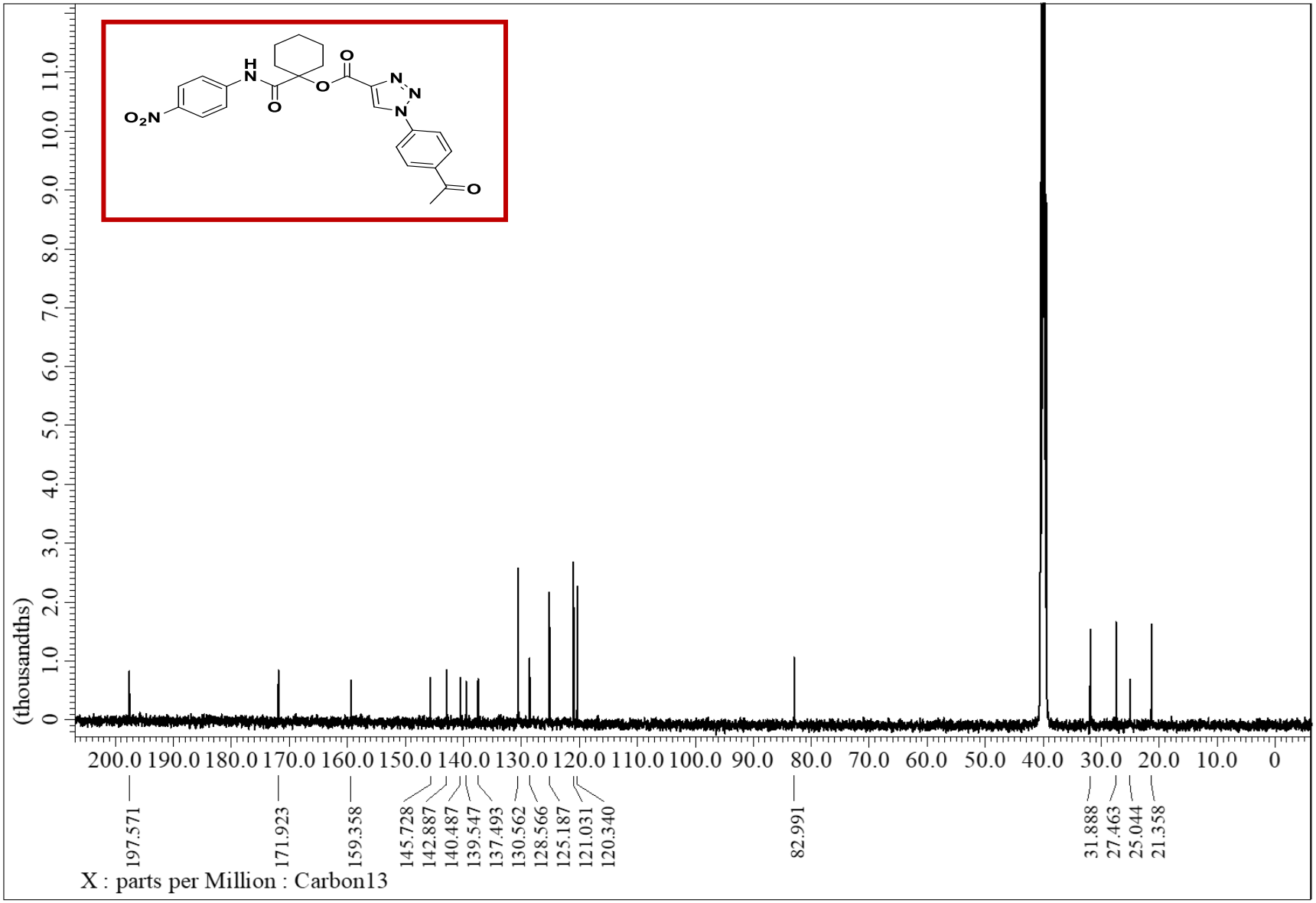


Figure S16. ^{13}C-NMR (125 MHz, DMSO-d_6) spectrum of **14**

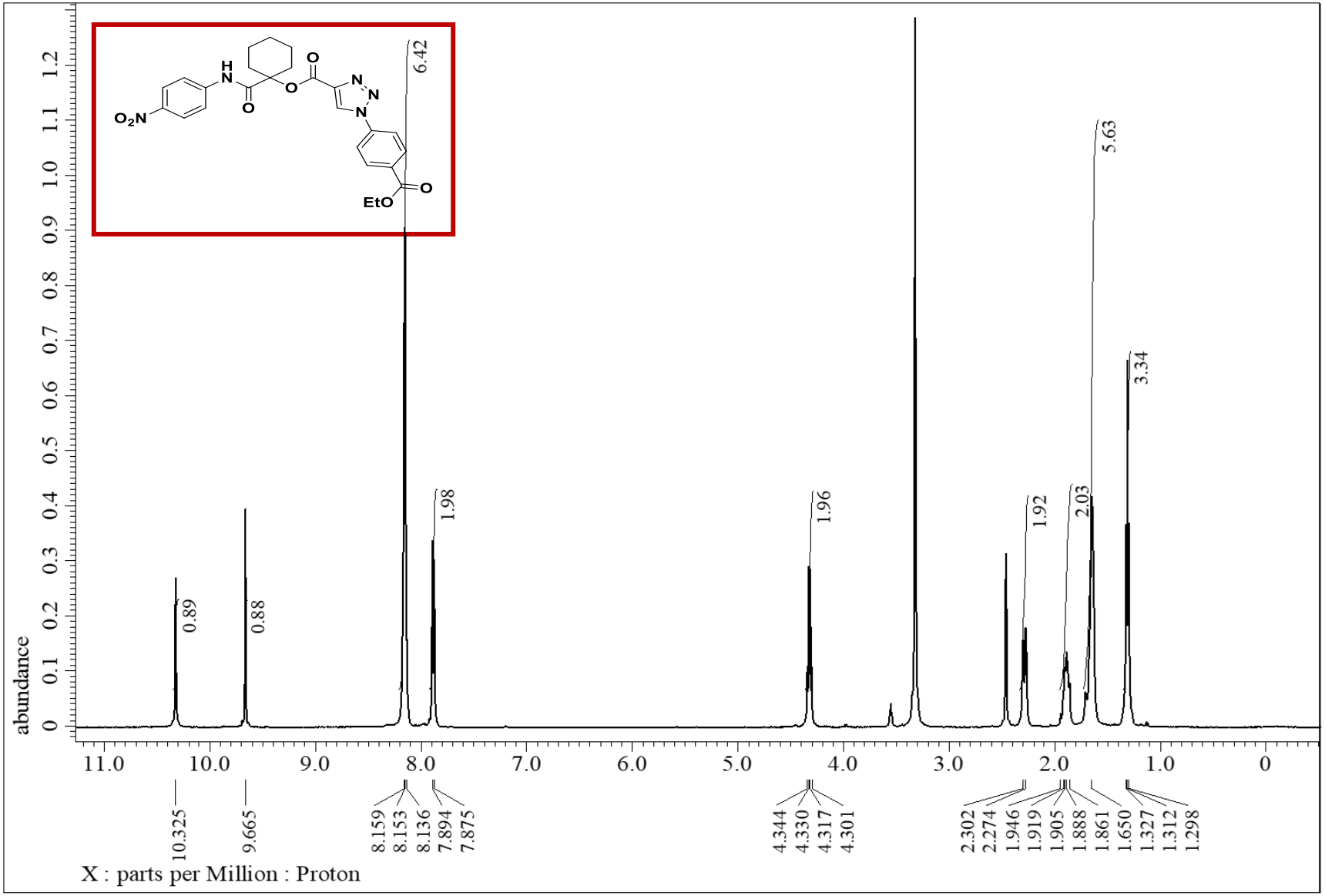


Figure S17. ^{1}H-NMR (500 MHz, DMSO-d_6) spectrum of **15**

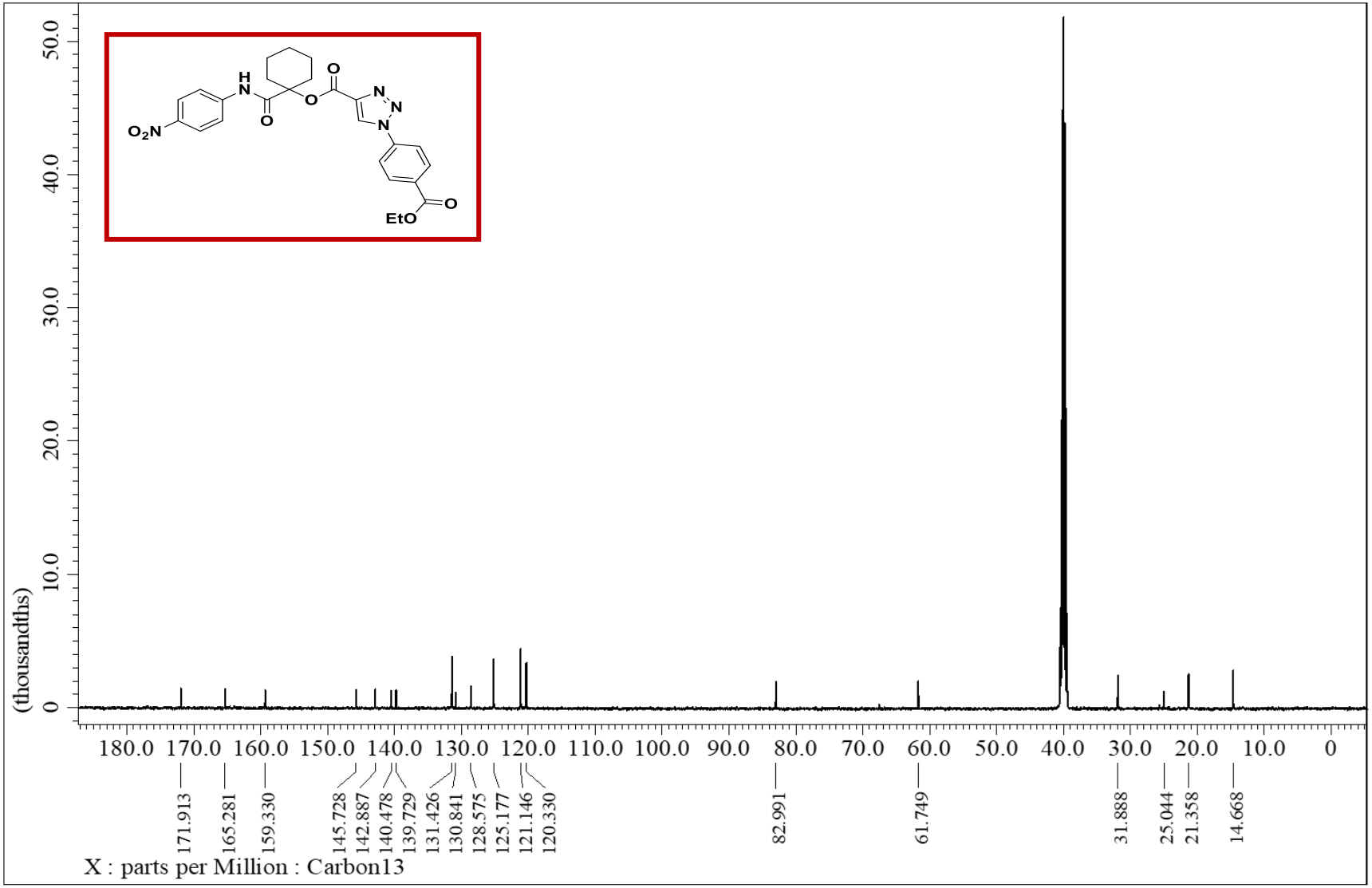


Figure S18. ^{13}C-NMR (125 MHz, DMSO-d_6) spectrum of **15**

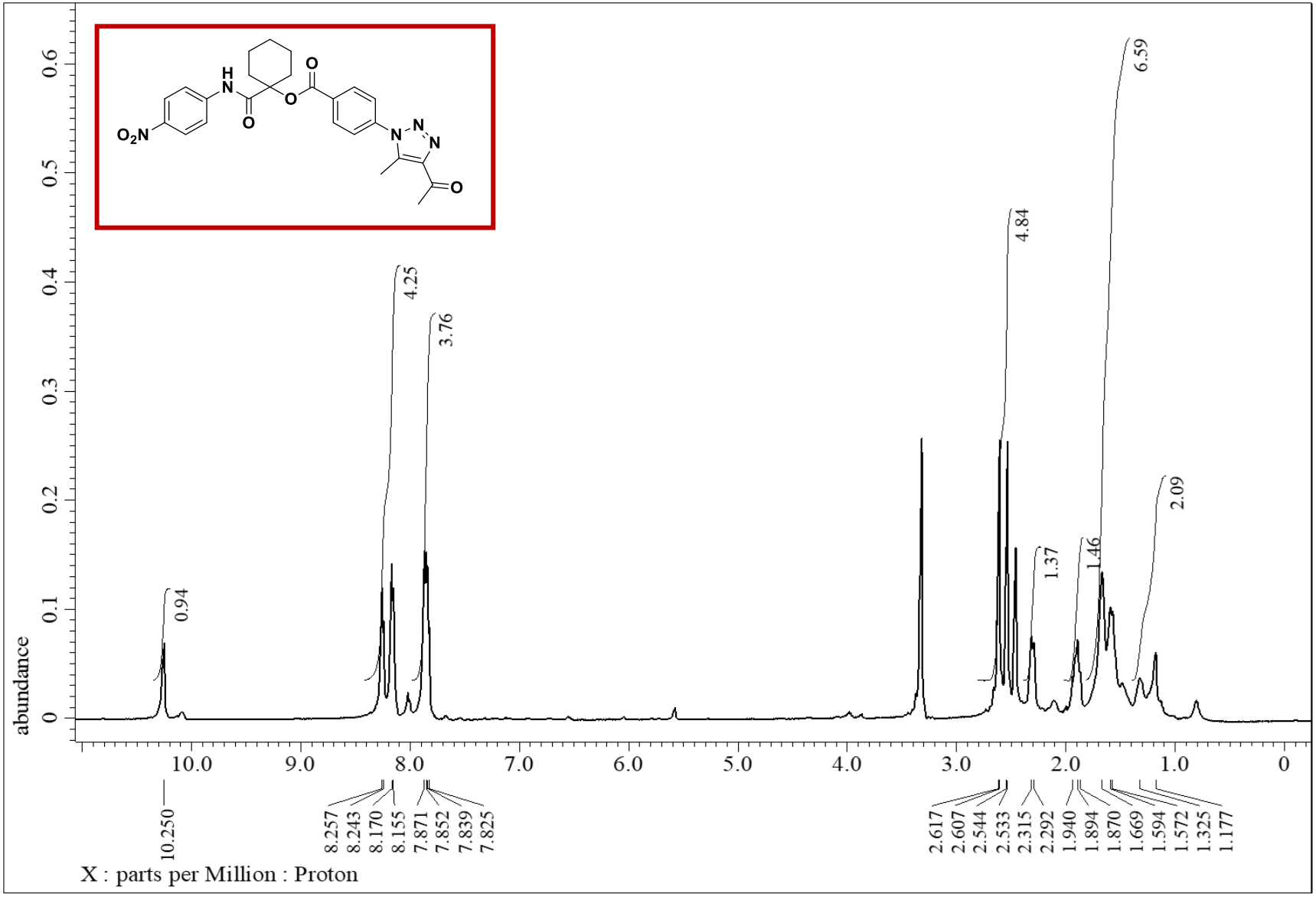


Figure S19. ^{1}H-NMR (500 MHz, DMSO-d_6) spectrum of **17**

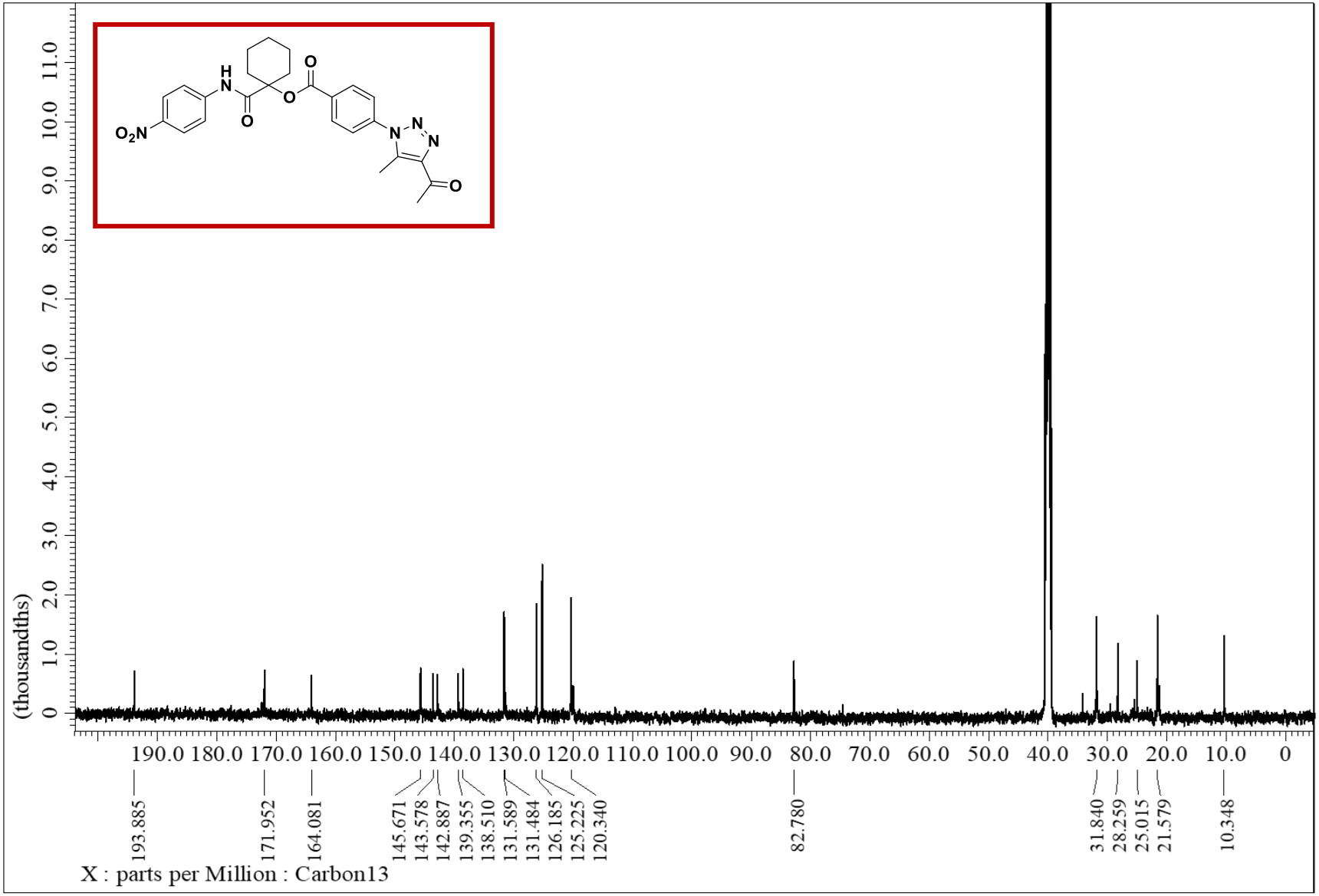


Figure S20. ^{13}C-NMR (125 MHz, DMSO-d_6) spectrum of **17**

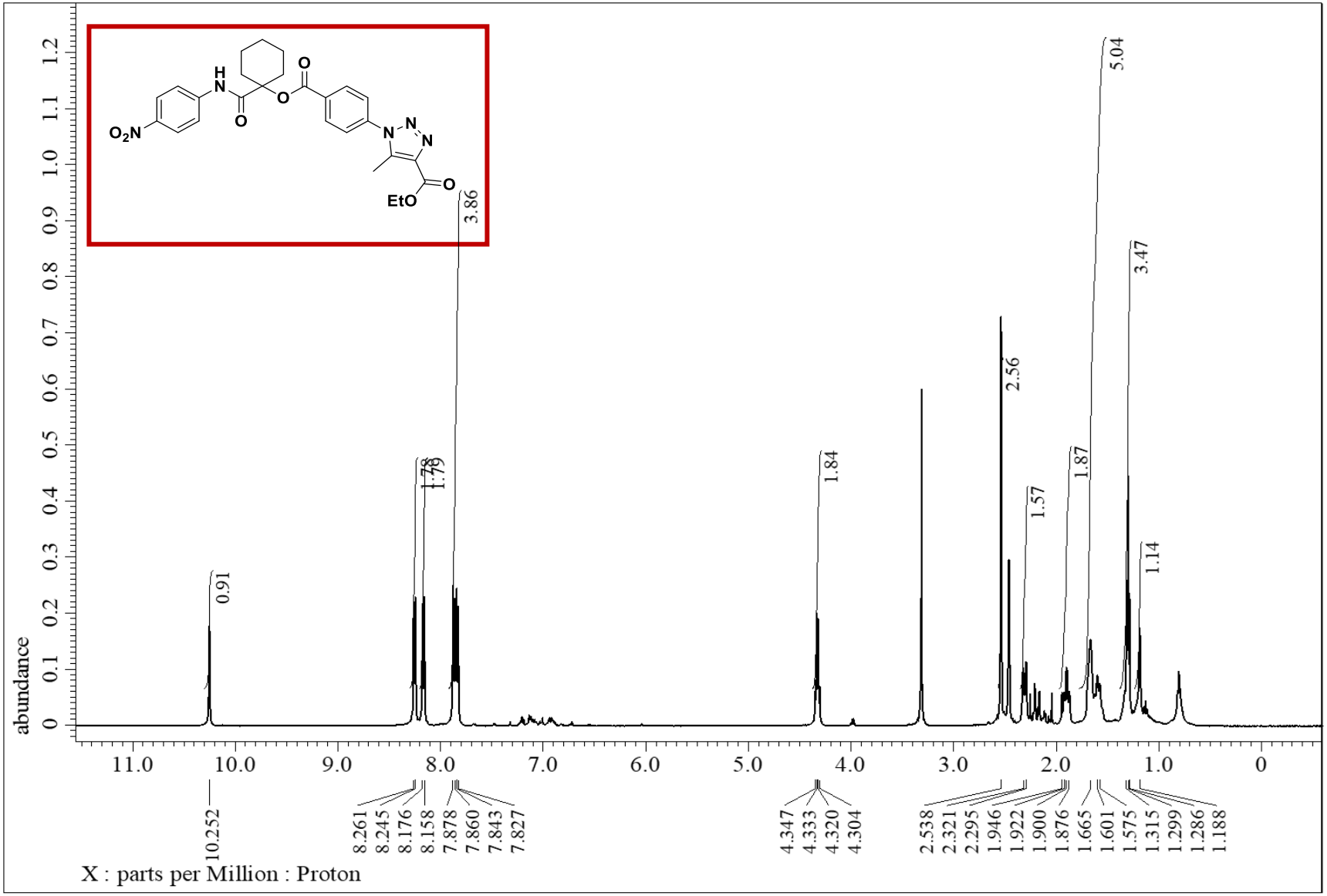


Figure S21. ^{1}H-NMR (500 MHz, DMSO-d_6) spectrum of **18**

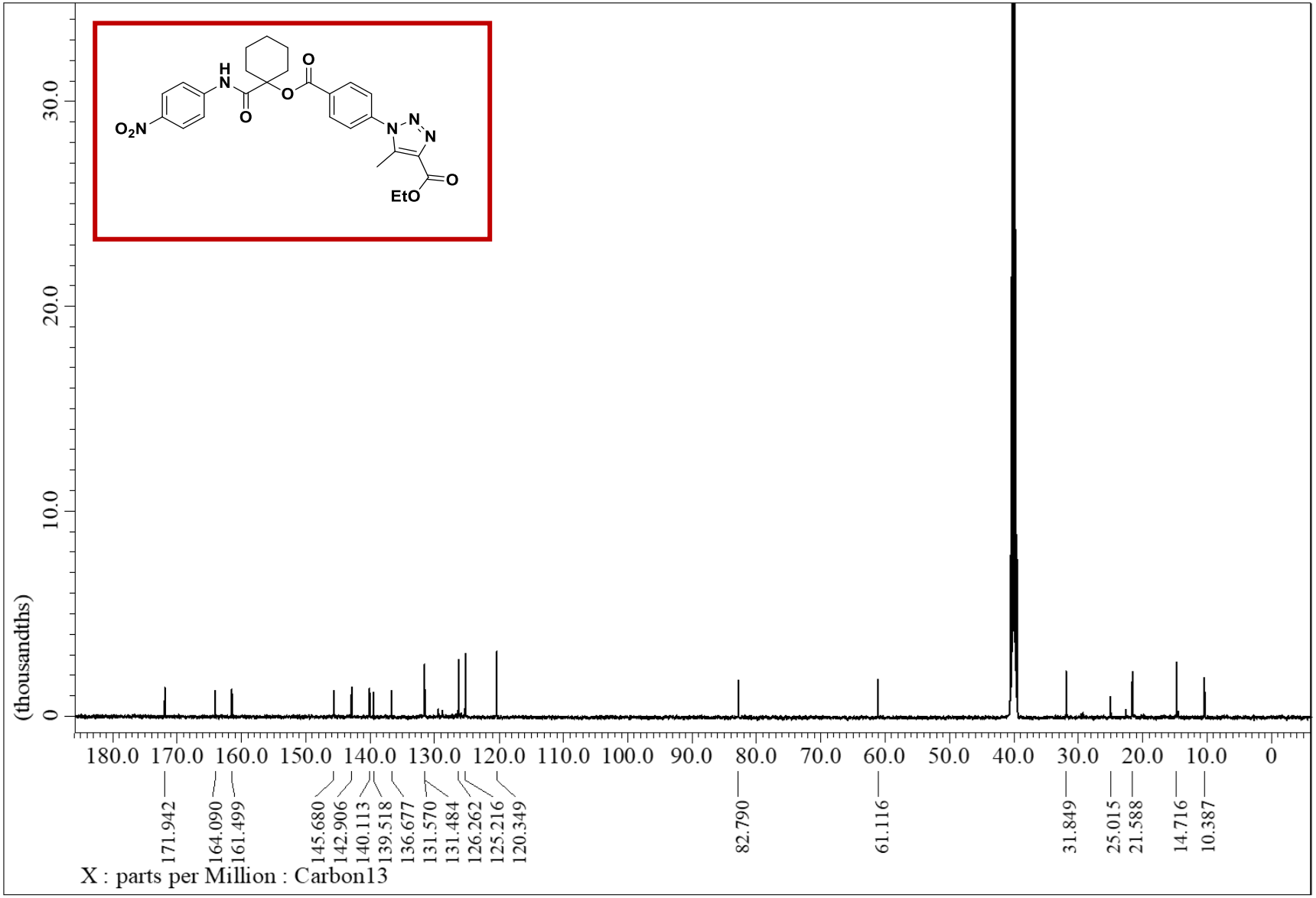


Figure S22. ^{13}C-NMR (125 MHz, DMSO-d_6) spectrum of **18**

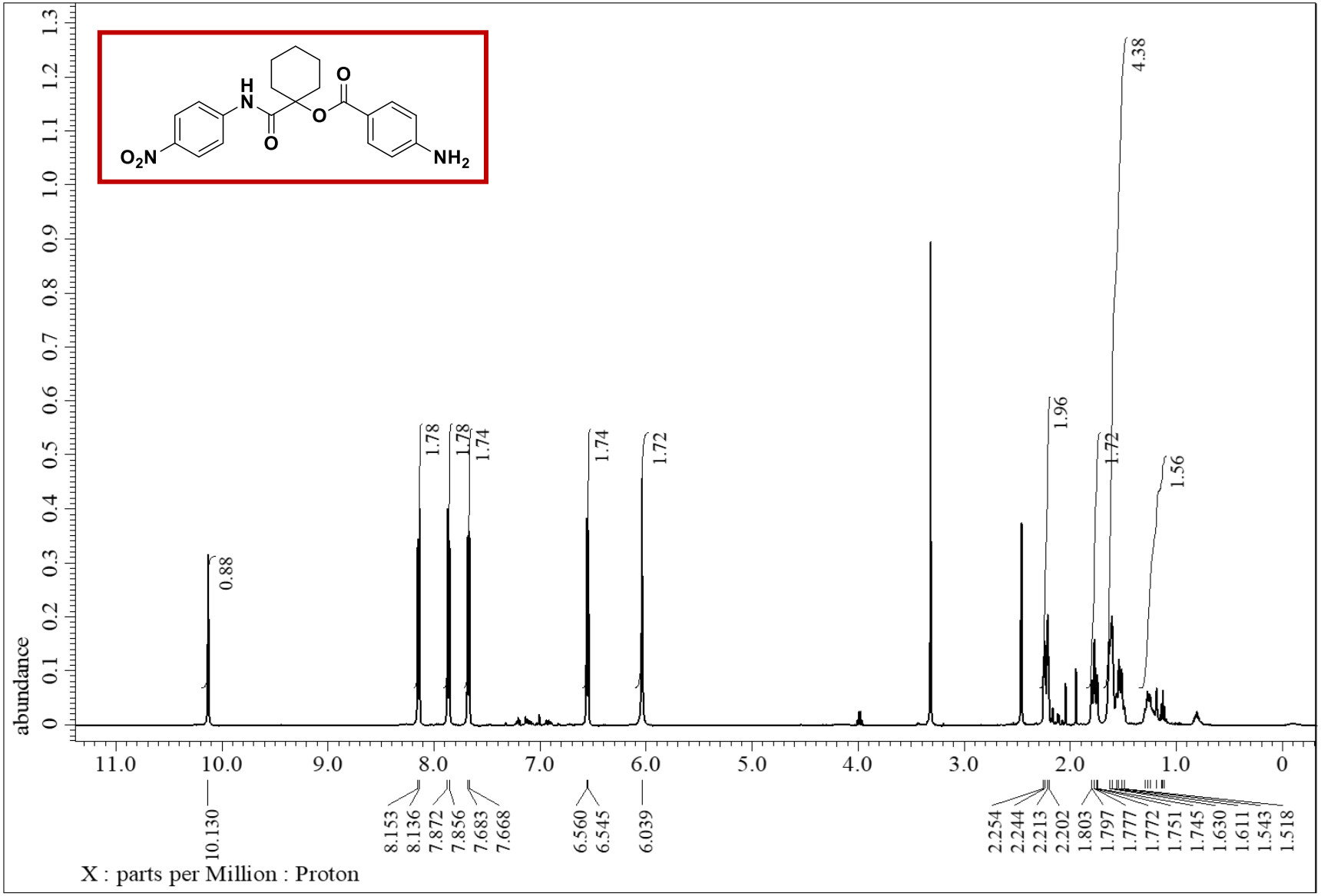


Figure S23. ^{1}H-NMR (500 MHz, DMSO-d_6) spectrum of **19**

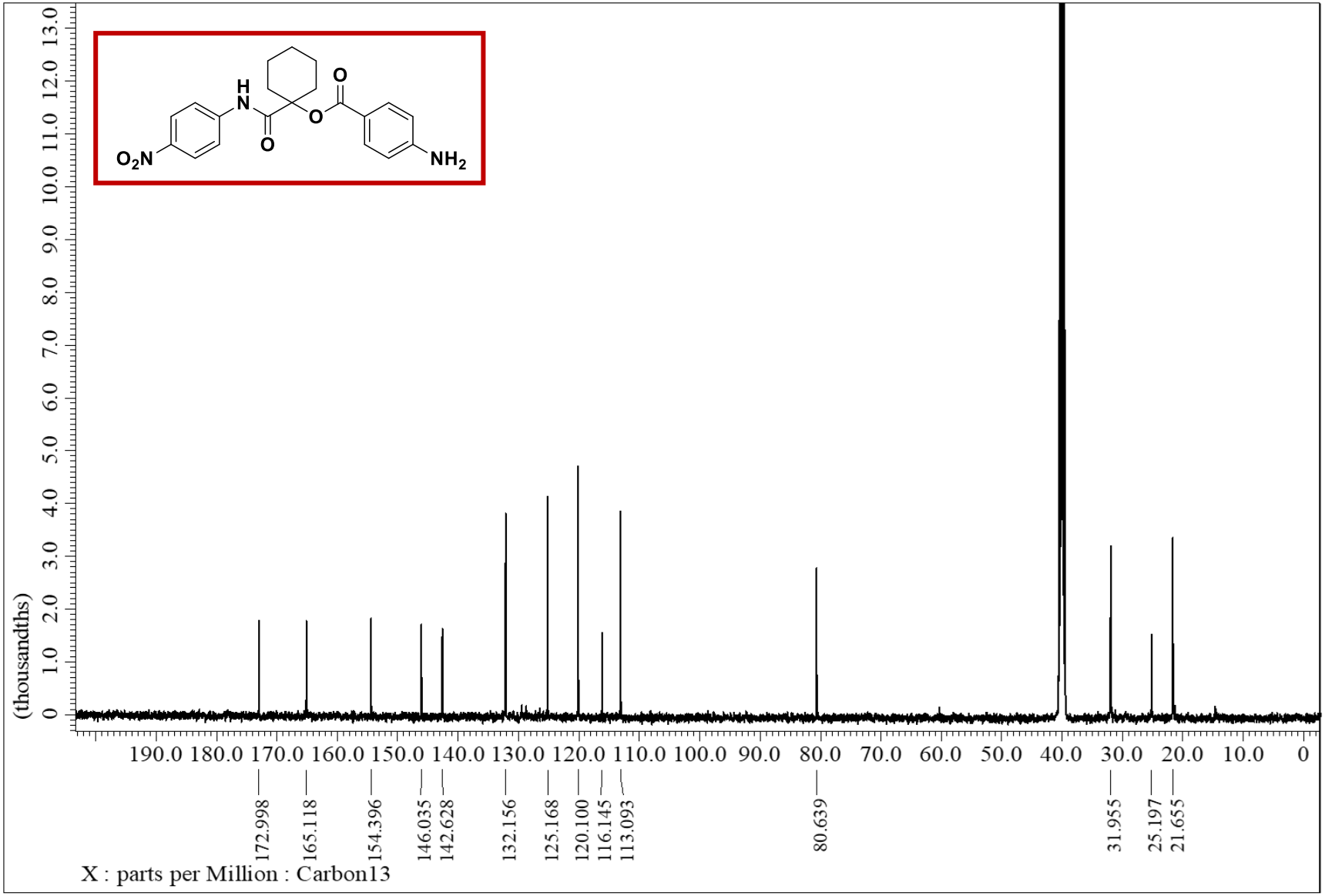
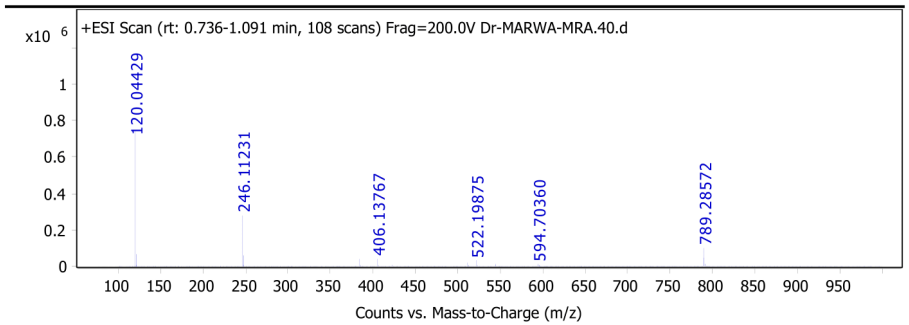


Figure S24. ^{13}C-NMR (125 MHz, DMSO-d_6) spectrum of **19**



Peak List

m/z	z	Abund
120.04429	1	747748.13
121.04732	1	68288.7
246.11231	1	278581.03
247.11423	1	56853.86
384.15567		38252.55
406.13767		41608.42
512.19314		22360.84
522.19875		31242.13
789.28572	1	102147.34
790.28881	1	47850.81

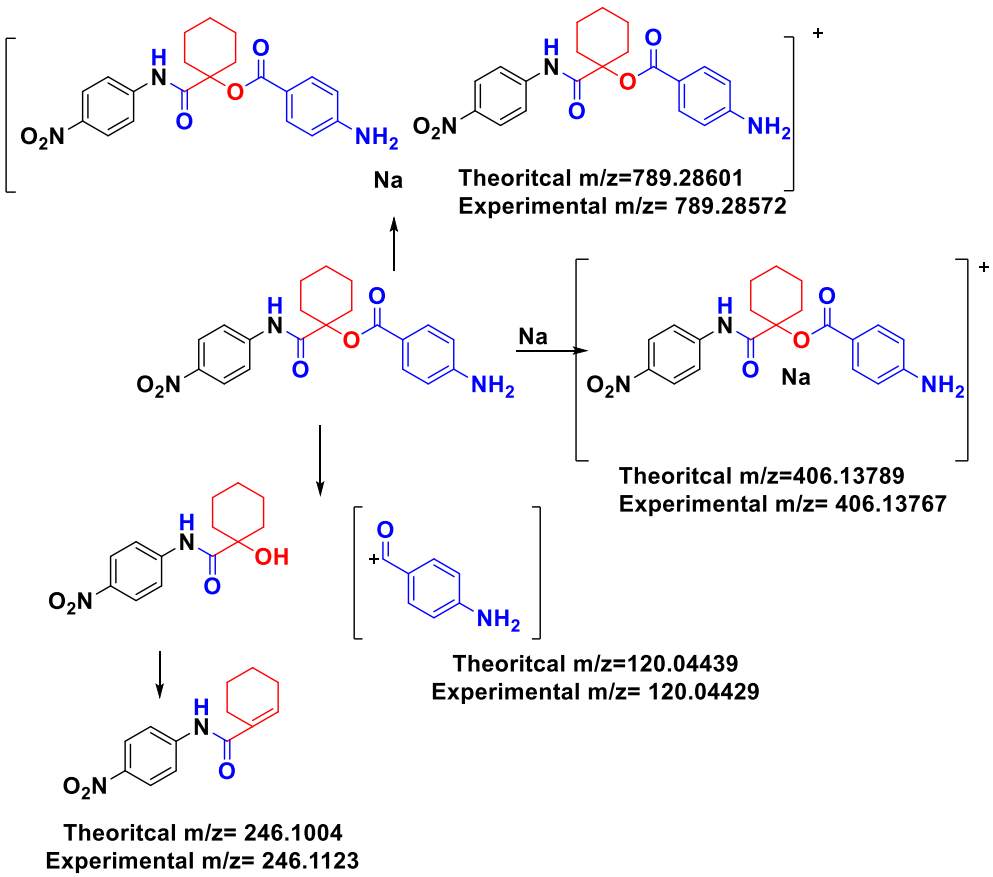


Figure S25. HRMS (ESI$^+$) of **19**

2. Material and Equipment

All starting compounds were commercially purchased. Reactions were performed in dried glassware. NMR spectra were recorded on a 400 or 500 MHz JEOLJNM ECA spectrometer. ^{13}C NMR spectra were obtained at either 100 or 125 MHz. Chemical shifts (δ) were reported in ppm. Melting points were recorded using Thermo Scientific1002D apparatus (220-240V, 200W, 50/60 Hz) and were uncorrected. Mass spectra were obtained using a direct probe controller inlet attached to a single quadrupole mass analyzer in a Thermo Scientific EIMS, Model: ISQ LT, using Thermo X-Calibur software. Values were reported as the ratio of mass to charge (m/z) in Daltons. IR spectra [ν_{max} /cm^{-1}] were recorded using PerkinElmer; FT-IR Spectrum BX and Bruker tensor 37 FT-IR.

3. Biological Evaluation

3.1. Evaluating the antioxidant activity

3.1.1. Thiobarbituric Acid Reactive Substances (TBARS) assay

Two milliliters of the test sample (1, 5, 10, 20, and 50 µg/mL), DMSO (control), or distilled water (blank) were incubated with an equal volume of liver homogenate (10% in phosphate buffer saline, pH 7.4) at 37°C for 45 minutes. Lipid peroxidation was induced by adding hydrogen peroxide (H_2O_2) and ferrous sulfate ($FeSO_4 \cdot 7H_2O$) to the reaction mixtures at final concentrations of 1 mM and 0.5 mM, respectively. After 30 minutes of incubation at 37°C, the reaction was stopped by adding butylated hydroxytoluene (BHT) at a final concentration of 0.02% and mixing gently. The mixtures were centrifuged at 3000 rpm for 15 minutes. One milliliter of the resulting supernatant was combined with 1 mL of 15% trichloroacetic acid (TCA) and centrifuged again at 3000 rpm for 10 minutes to remove proteins. The protein-free supernatant (1 mL) was then mixed with 500 µL of 0.7% thiobarbituric acid (TBA) and heated in a boiling water bath for 45 minutes. After cooling, the absorbance of the colored supernatant was measured at 532 nm.[1]. The scavenging activity was determined using the formula $[(A_C - A_E)/A_C] \times 100$, where A_C is the mean absorbance of the negative control and A_E is the mean absorbance of the sample. After that IC_{50} values were determined using the IC_{50} online calculator.[2]

3.1.2. Nitric Oxide (NO) scavenging assay

The method for determining nitric oxide (NO) scavenging activity is based on the ability of sodium nitroprusside to generate NO in an aqueous solution at physiological pH. This NO then reacts with oxygen to form nitrite ions, which can be quantified using the Griess reagent.[3] The scavenging activity of the sample is determined by measuring its ability to inhibit NO production, as previously described.[4]

Briefly, to a 96-well plate a volume of 50 µL of serial concentrations of the test compounds (10, 50, 100, 200, and 500 µg/mL) alongside distilled water as a negative control were added. To each well, 50 µL of 10 mM sodium nitroprusside solution was added, and the plate was incubated under light at room temperature for 90 minutes. Following incubation, an equal volume of Griess reagent was added to each well to react with the nitrite ions formed. The nitrite content was then measured immediately at 490 nm using an Optima spectrophotometer. The test was performed in triplicate to ensure accuracy and reliability of the results. The percentage of NO scavenging activity and the IC_{50} values were calculated as mentioned before.

3.1.3. Free radical scavenging activity by DPPH• (2, 2-diphenyl-1-picrylhydrazyl radical)

The scavenging activity of compounds against DPPH radicals was evaluated at room temperature using a UV-visible spectrophotometer following the previously described procedure.[5] Briefly, a DPPH solution with a maximum absorption at 519 nm in ethanol was prepared. Serial concentrations of the test compounds were added to the DPPH solution, and the mixtures were incubated for 30 minutes in the dark. Absorbance readings were recorded at 519 nm, and the percentage of scavenging activity was calculated based on the reduction in absorbance. IC_{50} values were calculated as previously described.

3.2. Evaluating the anti-inflammatory potential

3.2.1. In vitro 5-Lipoxygenase (5-LOX) inhibitory assay

The 5-LOX inhibition assay was conducted at room temperature following a previously described method.[6, 7] Briefly, the assay measured conjugated diene formation (HpETEs and HETEs) catalyzed by 5-LOX through absorbance at 234 nm using a UV–Vis spectrophotometer. The reaction buffer consisted of 50 mM Tris–HCl (pH 7.5), 0.3 mM $CaCl_2$, 0.1 mM EDTA, 0.1 mM ATP, and 40 µM arachidonic acid. All reactions were performed in 1-cm quartz cuvettes with a total buffer volume of 500 µL, and absorbance at 234 nm was continuously monitored for 300 seconds. The experiments were done in triplicate. Quercetin was utilized as positive control. The % inhibitions of the test compounds were obtained after successive dilutions and the IC_{50} values were then calculated.

3.2.2. Membrane stabilization (RBC's) assay

A serial dilution of test compounds and diclofenac (1, 5, 10, 20, and 50 µg/mL) was prepared in 0.9% NaCl. To each dilution, 900 µL was mixed with 100 µL of a 1% human red blood cell (RBC) suspension in a microtube. The mixtures were incubated at 37°C for 1 hour, followed by centrifugation at 3000×g for 5 minutes. After centrifugation, 200 µL of the supernatant was transferred to a 96-well plate, and absorbance was measured at 540 nm using a microplate reader. Positive and negative controls were prepared by mixing 100 µL of RBC suspension with 900 µL of distilled water (100% hemolysis) or 0.9% NaCl (0% hemolysis), respectively.[8]

The percentage of hemolysis was calculated using the following formula:

$$\text{Hemolysis (\%)} = \frac{\text{Abs}_{\text{sample}} - \text{Abs}_{\text{C-}}}{\text{Abs}_{\text{C+}} - \text{Abs}_{\text{C-}}} \times 100$$

Where:

- **$\text{Abs}_{\text{sample}}$** is the absorbance of the tested compounds.
- **$\text{Abs}_{\text{C+}}$** is the absorbance of the positive control (100% hemolysis).
- **$\text{Abs}_{\text{C-}}$** is the absorbance of the negative control (0% hemolysis).

All samples were assayed in triplicate. The inhibitory concentration (IC_{50}) required to achieve 50% anti-hemolytic activity was determined in µg/mL then converted to µM.

4. Conformational analysis study

Table 1 Energetics (A.U.) of the studied conformers for **4** in gas phase.

Parameter	4a	ab	4c	4d
E	-1423.3687	-1423.3727	-1423.3734	-1423.3717
ZPVE	0.3801	0.3802	0.3801	0.3798
E_{tot}	-1422.9886	-1422.9925	-1422.9933	-1422.9920
ΔE (Kcal.mol^{-1})	2.9056	0.4682	0.0000	0.8170
H	-1422.9618	-1422.9659	-1422.9666	-1422.9651
G	-1423.0482	-1423.0517	-1423.0524	-1423.0517
S (cal.mol^{-1}K^{-1})	181.8550	180.5530	180.5660	182.2060
G (Kcal.mol^{-1})	-892976.2896	-892978.449	-892978.9000	-892978.468
K	79.8270	2.1300		2.0630
%	0.6370	23.8710	50.8450	24.6460

Table 2 Energetics (A.U.) of the studied conformers for **4** in DMSO.

Parameter	4a	4b	4c	4d
E	-1423.3842	-1423.3861	-1423.3872	-1423.3893
ZPVE	0.3793	0.3794	0.3793	0.3792
E_{tot}	-1423.0049	-1423.0067	-1423.0078	-1423.0100
ΔE (Kcal.mol^{-1})	3.2191	2.1026	1.3914	0.0000
H	-1422.9780	-1422.9800	-1422.9811	-1422.9832
G	-1423.0652	-1423.0662	-1423.0674	-1423.0701
S (cal.mol^{-1}K^{-1})	183.5720	181.5030	181.5600	183.0110
G (Kcal.mol^{-1})	-892986.9491	-892987.5766	-892988.2894	-892990.022
K	5.1559	4.1030	2.9070	1.0000
%	10.8860	13.6790	19.3070	56.1270

Table 3 Energetics (A.U.) of the studied conformers for **4** in ethanol.

Parameter	4a	4b	4c	4d
E	-1423.3837	-1423.3857	-1423.3867	-1423.3886
ZPVE	0.3793	0.3794	0.3794	0.3792
E_{tot}	-1423.0043	-1423.0062	-1423.0074	-1423.0094

ΔE (Kcal.mol^{-1})	3.1854	1.9793	1.2710	0.0000
H	-1422.9774	-1422.9795	-1422.9806	-1422.9825
G	-1423.0645	-1423.0657	-1423.0669	-1423.0695
S (cal.mol^{-1}K^{-1})	183.3030	181.4480	181.5770	183.1270
G (Kcal.mol^{-1})	-892986.5010	-892987.2760	-892988.006	-892989.646
K	195.7840	53.3390	15.6610	1.0000
%	0.4696	1.7236	5.8704	91.9364

5. Molecular docking study

The three-dimensional crystal structure of the 5-LOX enzyme (PDB: 6N2W)[9] with a resolution of 2.71 Å was retrieved from the Protein Data Bank (PDB). The docking study was conducted using MOE software (MOE 2020.09) as the computational tool.[10] Hydrogen atoms were first added using the Protonate 3D algorithm, which determined the protonation states of amino acid residues and applied partial charges to atoms. The compounds were generated using the builder tool, followed by energy minimization with the MMFF94x force field. The MOE Dock tool was then employed to dock the synthesized compounds into the active site, and the final ligand–enzyme poses were selected based on binding energy scores and ligand–receptor interactions.

6. Data analysis and statistics

The data are expressed as mean ± standard error of mean (SEM) and the significant values were considered at $p < 0.05$. One-way analysis of variance (ANOVA) by Tukey's test used for evaluating the difference between the mean values of the studied treatments.[11] The analysis was done for three measurements using SPSS software version 16.

7. References

[1] A.E. Abd El-Wahab, D.A. Ghareeb, E.E. Sarhan, M.M. Abu-Serie, M.A. El Demellawy, In vitro biological assessment of Berberis vulgaris and its active constituent, berberine: antioxidants, anti-acetylcholinesterase, anti-diabetic and anticancer effects, BMC complementary and alternative medicine, 13 (2013) 1-12.

[2] https://www.aatbio.com/tools/ic50-calculator; Accessed December 2024.

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