Supplementary Information

Application of bioorthogonal hetero-Diels–Alder cycloaddition of 5-arylidene derivatives of 1,3-dimethylbarbituric acid and vinyl thioether for imaging inside living cells

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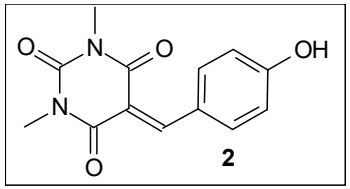
Experimental Section

Chemistry

Unless specified, all reagents and starting materials were purchased from commercial sources and used as received without purification. All reactions were monitored by thin-layer chromatography on 0.25 mm silica plates (F-254) visualized with UV light. Melting points were determined on a Boetius hot stage apparatus. IR spectra were measured using a Nicolet IR 200 FT-IR Thermo Scientific (with diamond stand) as film or powder. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$, DMSO-d_6, D_2O or CD_3CN on Bruker Avance II 600 MHz (^{1}H: 600 MHz, ^{13}C: 151 MHz) or 300 MHz (^{1}H: 300.18 MHz, ^{13}C: 75.48 MHz). Microanalyses were performed with Euro EA 3000 Elemental Analyzer or compounds purity was assessed by performing an HRMS (ESI-QTOF) spectra.

5-[(4-Hydroxyphenyl)methylidene]-1,3-dimethyl-1,3-diazinane-2,4,6-trione (2)

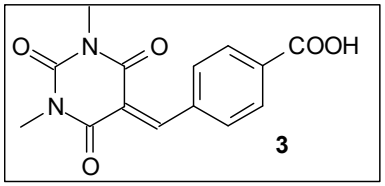


2

Compound **2** was prepared according to the procedure described in the literature[23]: To a stirred solution of 1,3-dimethylbarbituric acid **1** (0.465 g, 3 mmol) in water (8 mL) was added 4-hydroxybenzaldehyde (0.366 g, 3 mmol) rapidly and all at once. After 15 min of stiring at room

temperature, the solid product **2** was isolated by simple filtration and dried. Compound **2** needed no further purification and was obtained with 95% yield as a pale yellow solid.

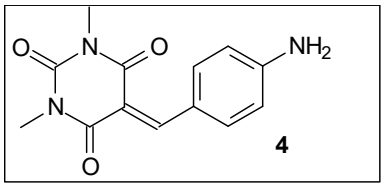
4-[(1,3-Dimethyl-2,4,6-trioxo-1,3-diazinan-5-ylidene)methyl]benzoic acid (3)



3

Compound **3** was obtained according to the procedure described in the literature[22]: Solid 1,3-dimethylbarbituric acid (0.465 g, 3 mmol), 4-carboxybenzaldehyde (0.448 g, 3 mmol) and amidosulfonic acid (0.298 g) were ground for 10 minutes with a mortal and pastel. It was then placed in a desiccator for 2 hours. The ground mixture was then dissolved in DMSO and poured into 50 mL distilled water. The formed precipitate was filtered, washed with boiling water and MeOH and dried in vacuum. Compound **3** needed no further purification and was obtained with 89% yield as a light yellow solid.

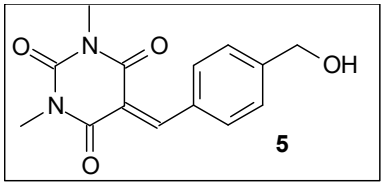
5-[(4-Aminophenyl)methylidene]-1,3-dimethyl-1,3-diazinane-2,4,6-trione (4)



4

p-Aminobenzaldehyde polymer (1.815 g, 15 mmol) and 100 mL of water were placed in a 150 mL round-bottomed flask on a magnetic stirrer. Then, to carry out the polymer hydrolysis, the mixture was acidified by adding approx. 5 mL conc. HCl. The suspension was stirred vigorously for 10 minutes until the polymer dissolved and hydrolyzed. 1,3-Dimethylbarbituric acid **1** (2.31 g, 15 mmol) was added to the solution with vigorously stirring. The mixture was then neutralized with saturated sodium bicarbonate solution, added gradually over about 15 min until the foaming mixture ceased. At the same time, the pH of the solution was checked to obtain pH = 7. The resulting dark red precipitate was filtered off and recrystallized from ethanol. Compound **4**: red solid, mp: > 360 °C (2.75 g, 67%); **IR** (powder) ν 3424, 3342, 3234, 1632, 1608, 1498, 1449, 1412, 1304, 1336, 1182, 1157, 1077 cm^{-1}; **^{1}H NMR** (600 MHz, DMSO-d_6) δ 8.33 (d, *J* = 9.0 Hz, 2H), 8.18 (s, 1H), 7.03 (br, 2H), 6.64 (d, *J* = 9.0 Hz, 2H), 3.22 (s, 6H); **^{13}C NMR** (151 MHz, DMSO-d_6) δ 163.8, 161.7, 157.2, 156.5, 151.7, 140.5, 120.6, 113.6, 109.0, 29.0, 28.3; **HRMS** (ESI-QTOF) m/z $[M+Na]^+$ calcd for $C_{13}H_{13}N_3NaO_3$ 282.0855, found 282.0849.

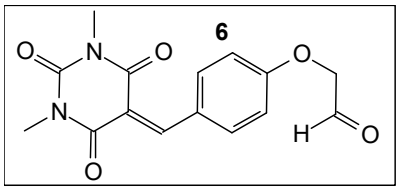
5-{[4-(Hydroxymethyl)phenyl]methylidene}-1,3-dimethyl-1,3-diazinane-2,4,6-trione (5)



To a stirred solution of 1,3-dimethylbarbituric acid **1** (0.465 g, 3 mmol) in water (10 mL) was added aldehyde **11** (0.405 g, 3 mmol) all at once. After 2 h of stiring at room temperature, the solid product **5** was isolated by simple filtration and dried. Compound **5** needed no further

purification: white solid, mp: 148-150 °C (0.694g, 85%); **IR** (powder) ν 3352, 2963, 2922, 1727, 1662, 1577, 1553, 1468, 1430, 1380, 1362, 1149, 1082 cm-1; **^{1}H NMR** (600 MHz, DMSO-d_6) δ 8.35 (s, 1H), 8.08 (d, *J* 7.5 Hz, 2H), 7.43 (d, *J* 7.5 Hz, 2H), 4.59 (s, 3H), 3.24 (s, 3H), 3.20 (s, 3H); **^{13}C NMR** (151 MHz, DMSO-d_6) δ 162.8, 160.9, 156.1, 151.6, 148.3, 133.9, 131.5, 126.2, 118.5, 63.0, 29.1, 28.5; **Anal. Calcd for** $C_{14}H_{14}N_2O_4$: C, 61.31; H, 5.14; N, 10.21. Found: C, 61.34; H, 5.19; N, 10.26.

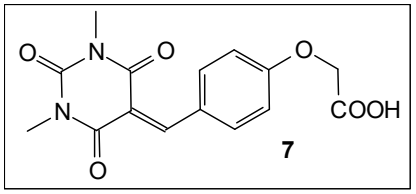
{4-[(1,3-Dimethyl-2,4,6-trioxo-1,3-diazinan-5-ylidene)methyl]phenoxy}acetaldehyde (6)



To a solution of acetal **15** (1.61 g, 4.3 mmol) in 15 mL of DCM, trifluoroacetic acid (9.92 g, 6.7 mL, 86 mmol) was added dropwise. The reaction mixture was stirred for 24 h at room temperature (monitored by TLC, hexane/ethyl acetate = 2:1). Then 50 mL of water was added and mixture was vigorously stirred. Precipitated yellow solid was filtered off and crystallized from ethanol. Compound **6**: yellow solid, mp: 159-161 °C, (1.18 g, 91%); **IR** (powder) ν 3452, 3108, 3063, 2966, 1651, 1568, 1542, 1442, 1365, 1271, 1200, 1115, 1085, 1041 cm^{-1}; **^{1}H NMR** (600 MHz, DMSO-d_6) δ 9.69 (s, 1H), 8.31 (s, 1H), 8.30 (d, *J*=8.3 Hz, 2H), 7.07 (d, *J*=7.0 Hz, 2H), 5.05 (s, 2H), 3.23 (s, 3H), 3.21 (s, 3H); **^{13}C NMR** (151

MHz, DMSO-d_6) δ 198.5, 163.2, 162.1, 161.2, 156.2, 156.0, 151.6, 137.9, 137.5, 126.2, 125.6, 116.3, 115.8, 114.9, 88.3, 73.1, 72.4, 29.1, 28.5; **HRMS** (ESI-QTOF) m/z $[M+Na]^+$ calcd for $C_{15}H_{14}N_2NaO_5$ 325.0800, found 325.0797.

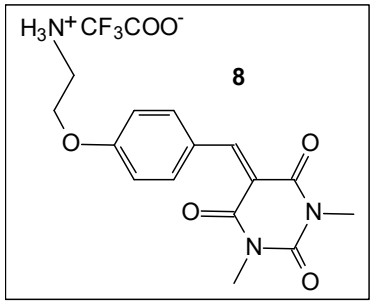
{4-[(1,3-Dimethyl-2,4,6-trioxo-1,3-diazinan-5-ylidene)methyl]phenoxy}acetic acid (7)



7

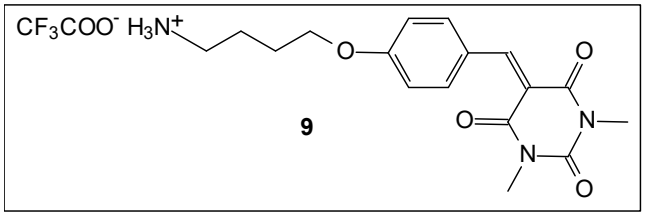
To a stirred solution of *N*,*N*-dimethylbarbituric acid **1** (1.56 g, 10 mmol) in 20 mL of water aldehyde **16** (1.80 g, 10 mmol) was added. The mixture was allowed to stir at 60 °C for 24 h, then the precipitated light-yellow solid was filtered off and washed with water, pale yellow solid (3.02 g, 95%); mp 212-216 °C; **IR** (powder) ν 3431, 1729, 1650, 1574, 1550, 1507, 1474, 1433, 1360, 1319, 1228, 1184, 1085 cm^{-1}; **^{1}H NMR** (600 MHz, DMSO-d_6) δ 8.32 (s, 1H), 8.29 (d, J = 9.0 Hz, 2H), 7.04 (d, J = 9.0 Hz, 2H), 4.84 (s, 2H), 3.23 (s, 3H), 3.21 (s, 3H); **^{13}C NMR** (151 MHz, DMSO-d_6) δ 170.1, 163.0, 162.3, 161.2, 156.0, 151.6, 137.5, 126.1, 116.2, 114.8, 65.0, 29.1, 28.5; **HRMS** (ESI-QTOF) m/z $[M+Na]^+$ calcd for $C_{15}H_{14}N_2NaO_6$ 341.0749, found 341.0742.

2-(4-((1,3-Dimethyl-2,4,6-trioxotetrahydropyrimidin-5(6H)-ylidene)methyl)phenoxy)ethylaminium 2,2,2-trifluoroacetate (8)



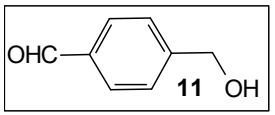
To a mixture of 6 mL of trifluoroacetic acid and 14 mL of DCM was added Boc-protected compound **25** (0.40 g, 0.99 mmol). The reaction mixture was stirred for 2 h at room temperature (monitored by TLC). Then it was brought to dryness by high vacuum. The crude product was redissolved in 1 mL of anhydrous methanol and 10 mL of anhydrous diethyl ether were added to precipitate the desired product **8**. Compound **8** was collected through filtration and dried under high vacuum as pale yellow solid, mp: 189-190 °C (335.0 mg, 87%); **IR** (powder) ν 3067, 2968, 1677, 1578, 1561, 1509, 1439, 1256, 1206, 1180, 1131 cm^{-1}; **^{1}H NMR** (300 MHz, DMSO-d_6) δ 8.34 (d, J = 9.0 Hz, 2H), 8.33 (s, 1H), 8.08 (br, 3H), 7.11 (d, J = 9.1 Hz, 2H), 4.30 (t, J = 5.0 Hz, 2H), 3.28 (m, 2H), 3.24 (s, 3H), 3.21 (s, 3H); **^{13}C NMR** (151 MHz, DMSO-d_6) δ 162.6, 161.8, 160.8, 158.2 (q, $J_{C\text{-}F}$ = 31.3 Hz), 155.5, 151.1, 137.1, 125.8, 115.9, 114.4, 64.8, 38.2, 28.7, 28.0; **HRMS** (ESI-QTOF) m/z [M-CF_3COO]$^+$ calcd for $C_{15}H_{18}N_3O_4$ 304.1297, found 304.1303.

2-(4-((1,3-Dimethyl-2,4,6-trioxotetrahydropyrimidin-5(6H)-ylidene)methyl)phenoxy)butylaminium 2,2,2-trifluoroacetate (9)



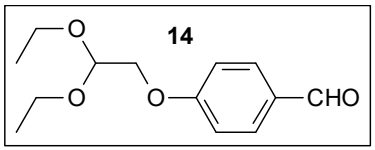
To a mixture of 6 mL of trifluoroacetic acid and 14 mL of DCM was added Boc-protected compound **26** (0.43 g, 0.99 mmol). The reaction mixture was stirred for 2 h at room temperature (monitored by TLC). Then it was brought to dryness by high vacuum. The crude product was redissolved in 1 mL of anhydrous methanol and 10 mL of anhydrous diethyl ether were added to precipitate the desired product **9**. Compound **9** was collected through filtration and dried under high vacuum as pale yellow solid, mp: 173-175 °C (374.6 mg, 85%); **IR** (powder) ν 2955, 1658, 1573, 1541, 1509, 1432, 1260, 1184, 1134 cm^{-1}; **^{1}H NMR** (300 MHz, DMSO-d_6) δ 8.32 (d, *J* = 9.0 Hz, 2H), 8.30 (s, 1H), 7.82 (br, 3H), 7.04 (d, *J* = 9.0 Hz, 2H), 4.12 (t, *J* = 5.0 Hz, 2H), 3.24 (s, 3H), 3.19 (s, 3H), 2.86 (m, 2H), 1.79 (m, 2H), 1.69 (m, 2H); **^{13}C NMR** (151 MHz, DMSO-d_6) δ 163.2, 163.1, 161.3, 156.2, 151.6, 137.9, 125.7, 115.9, 114.8, 67.9, 39.0, 29.1, 28.5, 25.9, 24.3; **HRMS** (ESI-QTOF) m/z [M-CF_3COO]$^+$ calcd for $C_{12}H_{22}N_3O_4$ 332.1610, found 332.1616.

4-(hydroxymethyl)benzaldehyde (11)



Alcohol **11** was synthesized by reduction of terephthalaldehyde using sodium borohydride[31]:To a suspension of terephthalaldehyde **10** (2.00 g, 14.92 mmol, 1 eq) in anhydrous ethanol (50 mL) in a 250 mL Erlenmeyer flask cooled to 0°C in an ice bath, powdered sodium borohydride (0.200 g, 5.22 mmol; 0.33 eq) was added in one portion, and the reaction continued to stir at 0°C. The reaction was allowed to stir for one hour, and was followed by TLC with 1:1 ethyl acetate and hexanes as the eluent. After 1 h, the reaction was warmed to room temperature and water (50 mL) and next ethyl acetate was added to this mixture, and it was vigorously shaken. The aqueous and organic layers were separated and the aqueous layer was extracted 3 × 20 mL with ethyl acetate. The combined organic layers were dried with $MgSO_4$ and evaporated under reduced pressure. Crude mixture was purified by flash chromatography (0 to 30% ethyl acetate/hexanes) to afford 91% of the **11** product as a white solid.

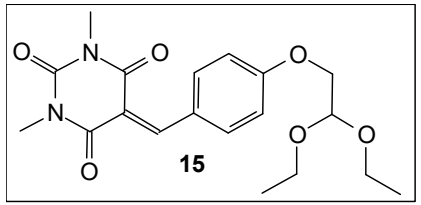
4-(2,2-diethoxyethoxy)benzaldehyde (14)



Compound **14** was synthesized according to a procedure described in the publication[26]: To a stirred suspension of 98% sodium hydride (2.5 g, 100 mmol) in DMF (100 mL) was added a solution of 4-hydroxybenzaldehyde **12** (10 g, 82 mmol) in DMF (100 mL) slowly dropwise at room temperature and stirred for 30 min and bromoacetaldehyde diethylacetal **13** (19.7 g, 100 mmol) was added to the reaction mixture, and the mixture was heated at 60 °C for 48 h. The reaction mixture was cooled to room temperature, quenched with water (200 mL), and extracted with

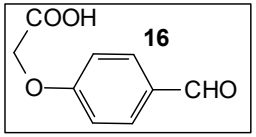
etyl acetate (3×300 mL). The combined organic layers were washed with brine, dried over $MgSO_4$, and concentrated. The crude compound was purified by chromatography using EtOAc/pet. ether (1:2) as eluent to yield the compound **14** (12.65 g, 58%) as a brown colored liquid.

5-{[4-(2,2-Diethoxyethoxy)phenyl]methylidene}-1,3-dimethyl-1,3-diazinane-2,4,6-trione (15)



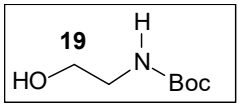
To a solution of 1,3-dimethylbarbituric acid **1** (1.0 g, 6.4 mmol) in water (25 mL) was added aldehyde **14** (1.77 g, 7.45 mmol). The reaction was carried out for 3 h at room temperature with vigorously stirring. The product **15** was collected through filtration and dried under high vacuum. Compound **15** was used in subsequent reaction without purification. **15**: yellow solid, mp: 130-132 °C (1.61 g, 67%); **IR** (powder) ν 3105, 3068, 2964, 1564, 1545, 1445, 1365, 1274, 1205, 1114, 1081, 1040 cm^{-1}; **^{1}H NMR** (600 MHz, $CDCl_3$) δ 8.44 (s, 1H), 8.24 (d, *J* = 9.0 Hz, 2H), 6.93 (d, *J* = 9.0 Hz, 2H), 4.79 (t, *J* = 5.0 Hz, 1H), 4.03 (d, *J* = 5.0 Hz, 2H), 3.71 (dq, *J* = 9.3, 7.0 Hz, 2H), 3.58 (dq, *J* = 9.3, 7.0 Hz, 2H), 3.34 (s, 3H), 3.32 (s, 3H), 1.18 (t, *J* = 7.0 Hz, 6H); **^{13}C NMR** (151 MHz, $CDCl_3$) δ 163.3, 163.1, 161.0, 158.9, 151.4, 137.9, 125.8, 114.6, 114.5, 100.2, 68.7, 62.9, 29.1, 28.4, 15.3; **Anal. Calcd for** $C_{19}H_{24}N_2O_6$: C, 60.63; H, 6.43; N, 7.44. Found: C, 60.69; H, 6.52; N, 7.51.

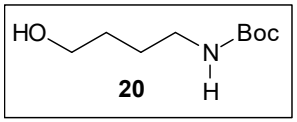
(4-formylphenoxy)acetic acid (16)



Compound **16** was synthesized according to a procedure described in literature[26]: To a stirred solution of *p*-hydroxybenzaldehyde **12** (1.22 g, 10 mmol) and bromoacetic acid (1.67 g, 12 mmol) in water (20 mL) was dropwise added 20 mL water solution of sodium hydroxide (0.96 g, 24 mmol) within 30 min at room temperature. Stirring of the reaction mixture continued for 1.5 h under reflux and then cooled to room temperature. The solution was acidified with concentrated hydrochloric acid to pH 1–2, and the resulting solid was filtered and recrystallized from 96% ethanol to give *p*-formylphenoxyacetyl acid as a white solid (1.40 g, 78%).

tert-Butyl (2-hydroxyethyl)carbamate (19) and tert-butyl (4-hydroxybutyl)carbamate (20)

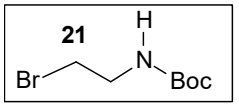


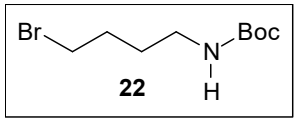


Compounds **19** and **20** were synthesized according to the procedure from the publication.[24] To 50 mL of anhydrous DCM was added di-*t*-butyl carbonate (3.57 g, 16.4 mmol). Ethanolamine **17** (1.00 g, 16.4 mmol) was added dropwise to the solution at 0 °C over 10 min. The reaction was

allowed to warm to room temperature and stirred for 2 h. The solvent was removed under reduced pressure and the crude product was purified via column chromatography with hexane and ethyl acetate (3:2, v/v) to yield the product **19** as colorless oil (98%).

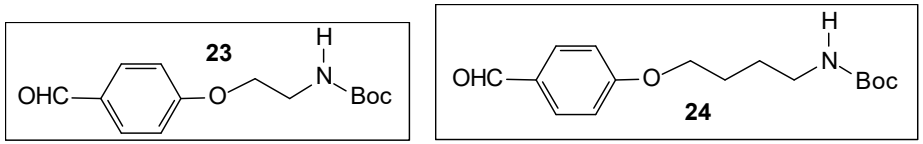
tert-Butyl (2-bromoethyl)carbamate (21) and tert-Butyl (4-bromobutyl)carbamate (22)





Alcohols **19** and **20** have been converted to bromides **21** and **22** via mesylate by the modified procedure described in literature[25]: A flask was charged with *tert*-butyl (2-hydroxyethyl)carbamate **19** (0.81 g, 5.0 mmol), methanesulfonyl chloride (0.47 mL, 6.0 mmol), and DCM (15 mL). To this stirring solution was added NEt_3 (0.9 mL, 6.5 mmol). Stirring was continued for 45 min, and then LiBr (4.35 g, 50 mmol) and acetone (15 mL) were added. The reaction mixture was stirred for an additional 24 h, and then the solvents were removed by rotary evaporation. The contents were partitioned between Et_2O (30 mL) and H_2O (20 mL), and the Et_2O layer was washed with brine, dried ($MgSO_4$), filtered, evaporated to dryness, and placed under vacuum to yield 1.09 g (4.95 mmol, 99%) of **21** as a white solid.

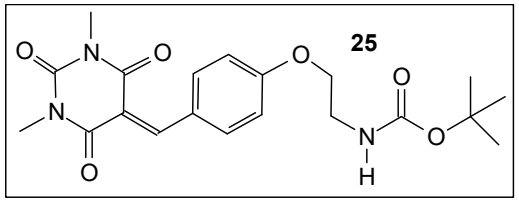
tert-Butyl [2-(4-formylphenoxy)ethyl]carbamate (23) and tert-Butyl [2-(4-formylphenoxy)butyl]carbamate (24)



Compounds **23** and **24** were synthesized according to a modified procedure from the publication[26]:

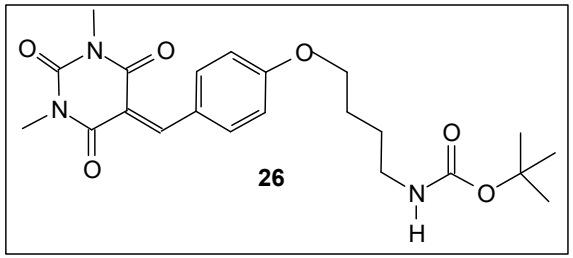
To a stirred suspension of 98% sodium hydride (0.25 g, 10 mmol) in DMF (10 mL) was added a solution of 4-hydroxybenzaldehyde (1 g, 8.2 mmol) in DMF (10 mL) slowly dropwise at room temperature and stirred for 30 min and bromide **21** (2.24 g, 10 mmol) was added to the reaction mixture, and the mixture was heated at 60 °C for 48 h. The reaction mixture was cooled to room temperature, quenched with water (20 mL), and extracted with etyl acetate (3·30 mL). The combined organic layers were washed with brine, dried over $MgSO_4$, and concentrated. The crude compound was purified by chromatography using EtOAc/pet. ether = 1:2 as eluent to yield the compound **23** (1.565 g, 59%) as a white solid.

tert-Butyl({4-[(2,4,6-trioxo-1,3-dimethyl-1,3-diazinan-5-ylidene)methyl]phenoxy}ethyl)carbamate (25)



To a solution of 1,3-dimethylbarbituric acid **1** (0.47 g, 3.00 mmol) in water (15 mL) was added aldehyde **23** (0.80 g, 3.00 mmol). The reaction was carried out for 24 h at 60 °C with vigorously stirring. After this time, the reaction mixture was cooled down to room temperature. The product **25** was collected through filtration and dried under high vacuum. Compound **25** was used in subsequent reactions without purification. **25**: yellow solid, mp: 129-130 °C (798.8 mg, 67%); **IR** (powder) 3372, 3014, 2982, 2930, 2870, 1725, 1689, 1665, 1567, 1533, 1458, 1437, 1281, 1177, 1153, 1086, 1055 cm^{-1}; **^{1}H NMR** (300 MHz, DMSO-d_6) δ 8.32 (d, *J* = 8.9 Hz, 2H), 8.31 (s, 1H), 7.06 (d, *J* = 9.0 Hz, 2H), 7.03 (s, 1H), 4.10 (t, *J* = 5.7 Hz, 2H), 3.35 (m, 2H), 3.23 (s, 3H), 3.21 (s, 3H), 1.39 (s, 9H); **^{13}C NMR** (151 MHz, DMSO-d_6) δ 162.7, 162.6, 160.8, 155.7, 151.1, 137.4, 125.2, 115.4, 114.3, 77.9, 66.9, 30.7, 28.6, 28.2, 28.0; **HRMS** (ESI-QTOF) m/z $[M+Na]^+$ calcd for $C_{20}H_{25}N_3NaO_6$ 426.1641, found 426.1635 .

tert-Butyl({4-[(2,4,6-trioxo-1,3-dimethyl-1,3-diazinan-5-ylidene)methyl]phenoxy}butyl)carbamate (26)

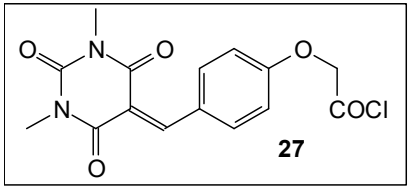


26

To a solution of 1,3-dimethylbarbituric acid (0.47 g, 3.00 mmol) in water (15 mL) was added aldehyde **24** (0.88 g, 3.00 mmol). The reaction was carried out for 24 h at 60 °C with vigorously stirring. After this time, the reaction mixture was cooled down to room temperature. The product **26**

was collected through filtration and dried under high vacuum. Compound **26** was used in subsequent reactions without purification. **26**: yellow solid, mp: 120-123 °C (866.7 mg, 67%); **IR** (powder) 3366, 2932, 1726, 1686, 1659, 1535, 1472, 1434, 1261, 1167, 1150, 1083, 1038 cm^{-1}; **^{1}H NMR** (300 MHz, DMSO-d_6) δ 8.43 (s, 1H), 8.24 (d, *J* = 8.9 Hz, 2H), 6.88 (d, *J* = 9.0 Hz, 2H), 4.57 (br, 1H), 4.01 (t, *J* = 5.7 Hz, 2H), 3.34 (s, 3H), 3.32 (s, 3H), 3.13 (m, 2H), 1.78 (m, 2H), 1.61 (m, 2H), 1.38 (s, 9H); **^{13}C NMR** (151 MHz, DMSO-d_6) δ 163.8, 163.2, 161.0, 159.0, 156.0, 151.5, 138.0, 125.5, 114.4, 79.3, 67.9, 40.2, 29.1, 28.45, 28.4, 26.8, 26.3; **HRMS** (ESI-QTOF) m/z $[M+Na]^+$ calcd for $C_{22}H_{29}N_3NaO_6$ 454.1954, found 454.1950.

{4-[(1,3-Dimethyl-2,4,6-trioxo-1,3-diazinan-5-ylidene)methyl]phenoxy}acetyl chloride (27)

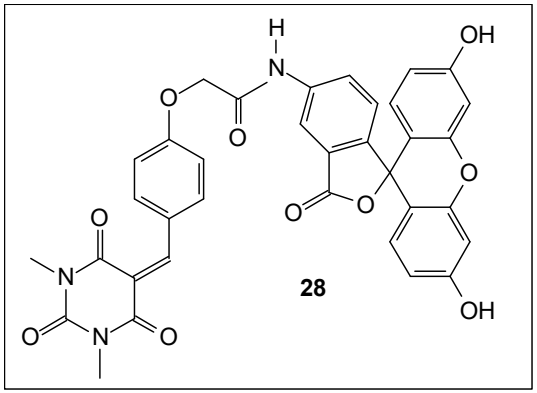


27

To a suspension of acetic acid derivative **7** (3.18 g, 10 mmol) in dry DCM (30 mL) oxalyl chloride (2.5 g, 1.7 mL, 20 mmol) and three drops DMF were added and stirred at room temperature overnight. The yellow suspension was filtered to obtain yellow solid product **27** which was dried under high vacuum (3.04 g, 90%); mp 170-175 °C; **IR** (powder) *ν* 3128, 3006, 2963, 2933, 1800, 1724, 1652, 1601, 1571, 1541, 1424, 1365, 1284, 1244, 1181, 1085 cm^{-1}; **^{1}H NMR** (600 MHz, DMSO-d_6) δ 8.32 (s, 1H), 8.30 (d, *J* = 9.0 Hz, 2H), 7.05 (d, *J* = 9.0 Hz, 2H), 4.84 (s,

2H), 3.23 (s, 3H), 3.21 (s, 3H); **^{13}C NMR** (151 MHz, DMSO-d_6) δ 170.0, 163.0, 162.3, 161.2, 156.0, 151.6, 137.5, 126.1, 116.2, 114.8, 66.1, 29.1, 28.5; **HRMS** (ESI-QTOF) m/z $[M+Na]^+$ calcd for $C_{15}H_{13}ClN_2NaO_5$ 359.0411, found 359.0419 .

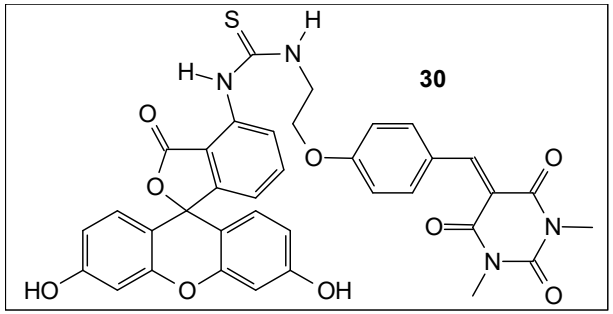
***N*-Fluoresceinylated-{4-[(1,3-dimethyl-2,4,6-trioxo-1,3-diazinan-5-ylidene)methyl]phenoxy}acetamide (28)**



To a stirred suspension of 5-aminofluorescein (0.1 g, 0.29 mmol) in glacial acetic acid (10 mL), chloride **27** (0.097g, 0.29 mmol) was added at room temperature and mixture was stirred for 30 min. Next, anhydrous sodium acetate (0.037 g, 0.45 mmol) was added and mixture was vigorously stirred at room temperature overnight. Afterwards to the reaction mixture water (20 mL) was added and suspension was stirred for 1 h. Finally, product **28** was filtered off and brought to dryness by high vacuum. Compound **28**: Dark red solid, mp: > 360 °C (156 mg, 84%); **IR** (powder) ν 3312, 1673, 1597, 1504, 1457, 1384, 1313, 1258, 1176, 1111, 1085 cm^{-1}; **^{1}H NMR** (600 MHz, DMSO-d_6) δ 10.62 (br, 1H), 10.00 (s, 2H), 8.31-8.34 (m, 3H), 7.89-7.93 (m, 2H), 7.13-7.25 (m, 3H), 6.52-6.67 (m, 6H), 4.93 (br, 2H), 3.23, 3.21 (s, 6H); **^{13}C NMR** (151 MHz,

DMSO-d_6) δ 191.9, 169.0, 167.1, 163.1, 160.0, 159.7, 152.4, 147.8, 140.4, 137.5, 132.3, 130.7, 129.6, 129.5, 127.4, 127.3, 125.0, 115.7, 115.0, 114.6, 113.1, 112.8, 110.1, 102.7, 83.6, 67.5, 29.1, 28.5; **HRMS** (ESI-QTOF) m/z $[M+H]^+$ calcd for $C_{35}H_{26}N_3O_{10}$ 648.1618, found 648.1615.

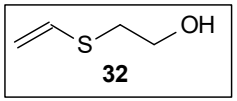
1-(3',6'-Dihydroxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-5-yl)-3-(2-(4-((1,3-dimethyl-2,4,6-trioxotetrahydropyrimidin-5(6*H*)-ylidene)methyl)phenoxy)ethyl)thiourea (30)



To a solution of fluorescein 5-isothiocyanate isomer (0.11 g, 0.28 mmol) in anhydrous DMF (1.5 mL) was added salt **8** (0.10 g, 0.24 mmol) in anhydrous DMF (1 mL). *N*,*N*-Diisopropylethylamine (0.13 mL, 0.72 mmol) was added dropwise subsequently. The reaction was stirred in the dark at room temperature for 24 h. The solvent was removed *in vacuo* and the crude product was purified by silica gel column chromatography ($CHCl_3$/MeOH=4:1) to give thiourea **30** as orange oil, (85.0 mg, 51%); **IR** (film) ν 3241, 2926, 1756, 1736, 1660, 1604, 1506, 1452, 1385, 1320, 1256, 1180, 1110 cm^{-1}; **^{1}H NMR** (300 MHz, DMSO-d_6) δ 10.21 – 9.82 (m, 3H), 8.46 – 8.16 (m, 3H), 7.89 (d, J = 8.6 Hz, 1H), 7.73 (d, J = 8.6 Hz, 1H), 7.30 – 7.12 (m, 2H), 6.86 (m, 1H), 6.73 – 6.50 (m, 5H), 4.63 – 4.23 (m, 2H), 4.18 – 3.80 (m, 2H), 3.40-3.30 (br, 2H), 3.27 – 3.09 (m,

4H); **^{13}C NMR** (151 MHz, DMSO-d_6) δ 191.4, 180.8, 168.5, 163.3, 159.5, 155.7, 151.9, 147.4, 141.2, 137.4, 131.9, 129.9, 129.1, 126.6, 124.2, 115.6, 115.1, 114.4, 112.6, 109.7, 102.3, 83.0, 79.2, 66.2, 43.1, 28.7, 28.3, 28.1, 26.3; **HRMS** (ESI-QTOF) m/z $[M+Na]^+$ calcd for $C_{36}H_{28}N_4NaO_9S$ 715.1475, found 715.1471.

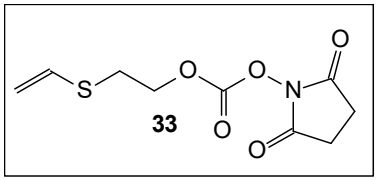
2-Vinylsulfanylethanol (32)



32

Compound **32** was prepared according to the procedure described in the literature[31]: To a stirred solution of sodium ethanoate in ethanol made by dissolving sodium (0.730 g, 31.8 mmol) in ethanol (15 mL), 2-mercaptoethanol **31** (1.985 g, 25.4 mmol) was added at room temperature. At 0 ºC the vinyl bromide solution (40 mL, 1M in THF, 40 mmol) was added and the reaction was transferred to an autoclave pipe, where was is heated to 105 ºC for 4 hours. After removal of solvent in vacuo, 15 mL water was added and the solution was extracted with 4 x 15 mL diethyl ether. The organic layer was dried with anhydrous magnesium sulfate and the solvent was removed in vacuo. This gave **32** (2.222 g, 84%), a brown oil with good purity as seen by ^{1}H NMR. A colorless oil was obtained by bulb-to-bulb distillation, aprox. bp. 110-125 ºC at 20-25 mbar. The structure of the compound **32** was confirmed by ^{1}H NMR spectrum (see ESI page S79): ^{1}H NMR (600 MHz, $CDCl_3$) δ 6.32 (dd, J = 16.8, J = 10.0 Hz, 1H), 5.25 (d, J = 9.8 Hz, 1H), 5.23 (d, J = 16.8, 1H), 3.80 (q, J = 5.8 Hz, 2H), 2.92 (t, J = 5.85 Hz, 2H), 2.05 (br, s, 1H).

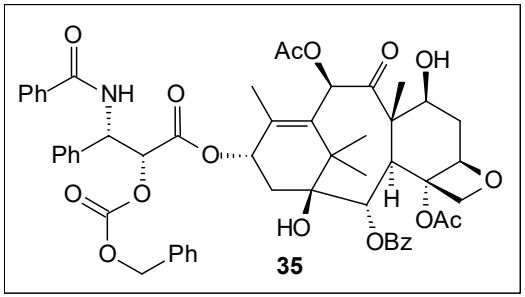
Succinimidyl 2-vinylsulfanylethyl carbonate (33)



33

Compound **33** was prepared according to the procedure described in the literature[31]: To a stirred solution of **32** (1.100 g, 10.56 mmol) in dry acetonitrile (40 mL), *N,N*'-disuccinimidylcarbonate (3.953 g, 15.43 mmol) was added at room temperature. To the reaction mixture, which was a cloud suspension, triethylamine (3.206 g, 31.7 mmol) was added, and after a couple of minutes the reaction mixture turns clear. Analysis by TLC (eluent: diethyl ether) shows that the reaction is complete after 30 min. The solvent was removed in vacuo. The residue was dissolved in 50 mL DCM and washed with 3 x 25 mL saturated $NaHCO_3$. The organic phase was washed with 30 mL brine and dried with anhydrous $MgSO_4$, and the solvent was removed in vacuo. The crude product was purified with flash column chromatography to obtain **33** (1.87 g, 7.6 mmol, 72%). The structure of the compound **33** was confirmed by ^{1}H NMR spectrum (see ESI page S80): ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.29 (dd, J = 10.0 Hz, J = 16.8 Hz, 1H), 5.29 (d, J = 10.0 Hz, 1H), 5.25 (d, J = 16.8 Hz, 1H), 4.45 (t, J = 7.2 Hz, 2H), 3.03 (t, J = 7.4 Hz, 2H), 2.84 (s, 4H).

2'-Carboxybenzoyltaxol (35)

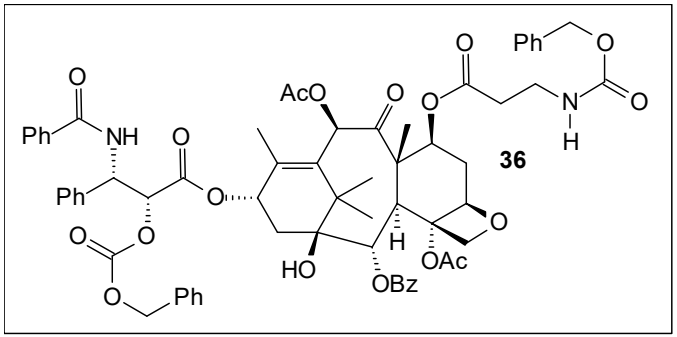


35

Compound **35** was prepared according to the procedure described in the literature[32]: A solution of taxol **34** (100 mg, 0.117 mmol) and pyridine (950 μL, 11.7 mmol, 100 eq.) in DCM (5 ml) at room temperature under argon was treated, at 10 min intervals, with benzyl chloroformate (170 μL aliquots, 1.17 mmol, 10 eq.). When thin layer chromatography (TLC) (EtOAc/hexane 1:1) showed no remaining starting material, the reaction was quenched with aqueous NH_4CI (1 mL). After dilution with ethyl acetale (10 mL), the reaction mixture was washed with ammonium chloride (2 x 5 mL), copper sulfate (4 x 10 mL), and sodium chloride (5 mL). The combined organic layer was dried ($MgSO_4$), concentrated, and purified by flash chromatography (EtOAc/hexane 1:1) to give 2'carboxybenzoyltaxol **35** as a white powder (80 mg, 70%). The structure of the compound **35** was confirmed by ^{1}H NMR spectrum (see ESI page S81): ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.14 (s, 3 H, 16-H) 1.25 (s, 3 H, 17-H) 1.69 (s, 3 H, 19-H), 1.76 (s, 1 H, l-OH), 1.93 (s, 3 H, 18-H), 1.90-1.94 (m, 1 H, 6β-H) 2.2-2.3, 2.4-2.5 (m, 2 H, 14-H), 2.24 (s, 3 H, 10-OAc-H), 2.46 (s, 3 H, 4-OAc-H), 2.51 (s, 1 H, 7-OH), 2.60 (m, 1 H, 6a-H), 3.82 (d, *J* = 7.0 Hz, 1 H, 3-H) 4.20 (d, *J*= 8.5 Hz, 1 H, 20-H), 4.32 (d, *J*= 8.5, 1 H, 20-H), 4.41 (m, 1 H, 7-H), 4.98 (d, *J* = 8.0 Hz, 1 H, 5-H) 5.18 (dd, *J*= 9.0 Hz, 10 Hz, 2 H, Bn), 5.45 (d, *J*= 2.5 Hz, 1 H, 2'-H), 5.69 (d, *J* = 7.0

Hz, 1 H, 2-H), 5.98 (dd, *J*= 2.5 Hz, 9.5 Hz, 1 H, 3'-H), 6.30 (br, 2 H, 10-H, 13-H), 6.92 (d, *J*= 9.5 Hz, 1 H, NH), 7.30-7.42 (m, 12 H, Ar-H), 7.44-7.54 (m, 3 H, Ar-H), 7.61 (t, *J* = 7.0 Hz, 1 H, Ar-H), 7.73 (d, *J* = 8.5 Hz, 2 H, Bz-H), 8.14 (d, *J* = 8.5 Hz, 2 H, Bz-H).

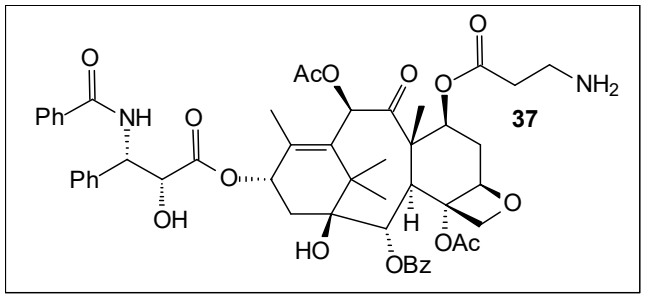
2'-Carboxybenzoyl-7-(*N*-carboxybenzoyl-*β*-alanyl)-taxol (36)



Compound **36** was prepared according to the procedure described in the literature[32]: A solution of 2'-carboxybenzoyltaxol **35** (25 mg, 0.025 mmol) and 4-(*N*,*N*-dimethylamino)pyridine (3 mg, 0.025 mmol, 1 eq.) in DCM (2 mL) at room temperature under argon was treated with N-carboxybenzoyl-β-alanine (24 mg, 0.11 mmol, 4.4 eq.) and dicyclohexylcarbodiimide (22 mg, 0.11 mmol, 4.4 eq.). After 18 h, the mixture was diluted with DCM (5 mL) and filtered to remove the urea. The crude product was evaporated to dryness *in vacuo* and purified by flash chromatography (40-45 % EtOAc in hexane) to give 2'-carboxybenzoyl- 7-(*N*-carboxybenzoyl-β-alanyl)-taxol as a white powder (28 mg, 95%). The structure of the compound **36** was confirmed by ^{1}H NMR spectrum (see ESI page S82): ^{1}H NMR (300 MHz, $CDCl_3$) δ 1 .15 (s, 3 H, 16-H) 1.21 (s, 3 H, 17-H), 1.81 (s, 3 H, 19-H), 1.99 (s, 3 H, 18-H) 2.06 (s, 3 H, 10-OAc-H), 2.21 (m, 1 H, 6β-H), 2.4-2.6 (m, 5 H, 14-H, 6α-H, alanyl

CH_2), 2.46 (s, 3 H, 4-OAc-H), 3.4-3.5 (m, 2 H, alanyl CH_2), 3.96 (d, *J* = 7.0 Hz, 1 H, 3-H), 4.20 (d, *J* = 8.5 Hz, 1 H, 20-H), 4.33 (d, *J* = 8.5 Hz, 1 H, 20-H), 4.95 (d, *J* = 8.5 Hz, 1 H, 5-H) 5.18 (dd, *J* = 9 Hz, *J* = 10 Hz, 2 H, Bn), 5.45 (d, *J* = 2.5 Hz, 1 H, 2'-H), 5.63 (dd, *J* = 5.0 Hz, *J* = 7.0 Hz, 1 H, 7-H), 5.70 (d, *J* = 7.0 Hz, 1 H, 2-H), 5.98 (dd, *J* = 2.5 Hz, *J* = 9.0 Hz, 1 H, 3'-H), 6.22-6.30 (m, 2 H, 10-H, 13-H), 6.98 (d, *J* = 9.0 Hz, 1 H, NH), 7.30-7.42 (m, 17 H, Ar-H), 7.43-7.52 (m, 3 H, Ar-H), 7.61 (t, *J* = 7.0 Hz, 1 H, Ar-H), 7.73 (d, *J* = 8.5 Hz, 2 H, Bz-H), 8.14 (d, *J* = 8.5 Hz, 2 H, Bz-H).

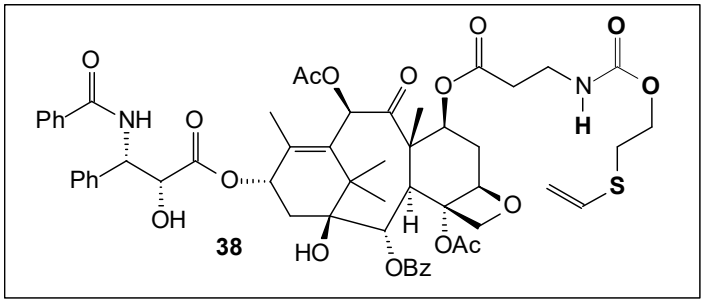
7-β-alanyltaxol (37)



Compound **37** was prepared according to the procedure described in the literature[32]: A solution of compound **36** (25 mg, 0.021 mmol) in methanol (1 mL) at room temperature under argon was treated with 10 % palladium on carbon (1 mg, 4 wt %) and placed under hydrogen. After 2 h, the mixture was filtered to remove the catalyst and evaporated to dryness *in vacuo* to give 7-β-alanyltaxol **37** as a white powder (19 mg, 98%). The structure of the compound **37** was confirmed by ^{1}H NMR spectrum (see ESI page S83): ^{1}H NMR (300 MHz, CD_3OD) δ 1.09 (s, 3 H,

16-H), 1.16 (s, 3 H, 17-H), 1.78 (s, 3 H, 19-H), 1.78-1.89 (m, 1 H, 6β-H), 1.87 (s, 3 H, 18-H), 1.91-2.00 (m, 1 H, 14-H), 2.18 (s, 3 H, 10-OAc-H), 2.19-2.24 (m, 1 H, 14-H), 2.38 (s, 3 H, 4-OAc-H), 2.50-2.59 (m, 1 H, 6α-H), 2.65-2.67 (m, 2 H, alanyl CH_2), 3.12-3.22 (m, 2 H, alanyl CH_2), 3.90 (d, J = 7.0 Hz, 1 H, 3-H) 4.19 (dd, J = 7.0 Hz, J = 14.0 Hz, 2 H, 20-H), 4.73 (d, J = 6.0 Hz, 1 H, 2'-H), 5.00 (d, J = 8.5 Hz, 1 H, 5-H), 5.63-5.65 (m, 3 H, 2-H, 3'-H, 7-H), 6.14 (br t, J = 6.0 Hz, 1 H, 13-H), 6.19 (s, 1 H, 10-H), 7.28-7.67 (m, 11 H, Ar-H, NH), 7.85 (dd, J = 1.5 Hz, J = 7.5 Hz, 2 H, Bz-H), 8.14 (dd, J = 1.5 Hz, J = 7.5 Hz, 2 H, Bz-H).

Vinyl thioether taxol (38)

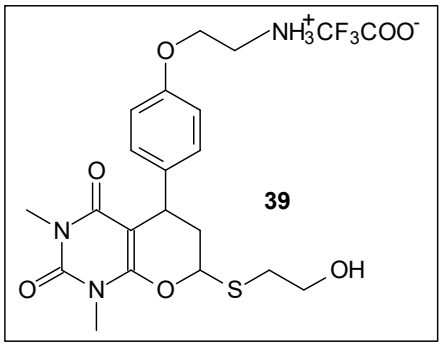


38

Compound **38** was prepared according to the procedure described in the literature[9]: To a solution of 7-β-alanyltaxol **37** (30 mg, 0.032 mmol) in anhydrous acetonitrile (2 mL) was added compound **33** (12 mg, 0.048 mmol) in anhydrous acetonitrile (1 mL) at room temperature. After 24 h, the mixture was evaporated to dryness *in vacuo* and purified by flash chromatography (silica gel, 40% EtOAc in petrol ether) to afford the desired product **38** as a white power (20 mg, 59%). The structure of the compound **38** was confirmed by ^{1}H NMR spectrum (see ESI page S84):

^{1}H NMR (300 MHz, $CDCl_3$) δ 1.16 (s, 3H), 1.21 (s, 3H), 1.81 (s, 3H), 1.83 (s, 3H), 1.78-1.86 (m, 2H), 2.18 (s, 3H), 2.33 (d, *J* = 9.2 Hz, 2H), 2.38 (s, 3H), 2.52-2.58 (m, 3H), 2.92 (t, *J* = 7.2 Hz, 2H), 3.44 (m, 2H), 3.65 (d, *J* = 5.2 Hz, 1H), 3.92 (d, *J* = 6.4 Hz, 1H), 4.17-4.23 (m, 3H), 4.31 (d, *J* = 8.4 Hz, 1H), 4.80 (m, 1H), 4.92 (d, *J* = 8.8 Hz, 1H), 5.21 (m, 2H), 5.58 (m, 1H), 5.67 (d, *J* = 6.4 Hz, 2H), 5.79 (dd, *J* = 8.8 Hz, 2.0 Hz, 1H), 6.18 (t, *J* = 8.4 Hz, 1H), 6.24 (s, 1H), 6.33 (dd, *J* = 16.8 Hz, 9.6 Hz, 1H), 7.07 (d, *J* = 8.8 Hz, 1H), 7.33-7.64 (m, 11H), 7.76 (d, *J* = 7.6 Hz, 2H), 8.11 (d, *J* = 8.0 Hz, 2H).

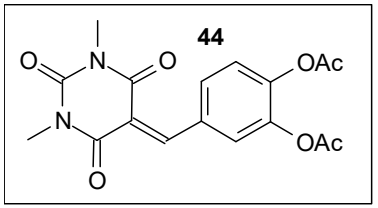
(4-{7-[(2-Hydroxyethyl)sulfanyl]-1,3-dimethyl-2,4-dioxo-1,3,4,5,6,7-hexahydro-2*H*-pyrano[2,3-*d*]pyrimidin-5-yl}phenoxy)ethanaminium 2,2,2-trifluoroacetate (39)



To a solution of heterodiene **8** (0.035 g, 0.084 mmol) in water (2 mL) was added thioether **32** (0.0875 g, 0.84 mmol). The reaction was stirred at 37 °C for 24 h (monitored by TLC, $CHCl_3$/MeOH = 2:1). The solvent was removed *in vacuo* and the crude product was purified by silica gel column chromatography (DCM/MeOH = 2:1) to give **39** as pale yellow oil (23.2 mg, 53%); **IR** (neat) ν 3390, 2952, 1682, 1634, 1509, 1248,

1205, 1140, 1023 cm^{-1}; **^{1}H NMR** (300 MHz, DMSO-d_6) δ 8.13 (s, 4H), 7.16 (d, J = 8.7 Hz, 2H), 6.89 (d, J = 8.6 Hz, 2H), 5.39 (dd, J = 10.3, 2.4 Hz, 1H), 4.91 (t, J = 5.5 Hz, 1H), 4.23 – 4.13 (m, 2H), 4.00 – 3.91 (m, 2H), 3.67 – 3.47 (m, 4H), 3.31 (s, 3H), 3.11 (s, 3H), 2.87 – 2.80 (m, 2H); **^{13}C NMR** (151 MHz, DMSO-d_6) δ 161.4, 157.9 (d, $J_{C\text{-}F}$ = 30.8 Hz), 156.5, 156.0, 150.6, 136.8, 128.7, 118.3, 116.3, 114.5, 87.6, 82.0, 64.4, 61.0, 38.4, 33.5, 33.0, 28.6, 27.5; **HRMS** (ESI-QTOF) m/z $[M\text{-}CF_3COO]^+$ calcd for $C_{19}H_{26}N_3O_5S$ 408.1593, found 408.1589.

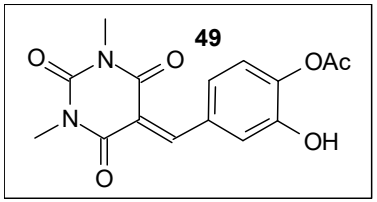
5-[(3,4-diacetoxyphenyl)methylidene]-1,3-dimethyl-1,3-diazinane-2,4,6-trione (44)



To a stirred solution of 1,3-dimethylbarbituric acid **1** (0.465 g, 3 mmol) in water (10 mL) was added 3,4-diacetoxybenzaldehyde **43** (0.666 g, 3 mmol). After 24h of stiring at room temperature, the solid product **44** was isolated by filtration and dried. Compound **44** needed no further purification and was obtained with 95% yield. Compound **44**: white solid, mp 149-150 °C; **IR** (powder) ν 3012, 2968, 2930, 1773, 1735, 1670, 1654, 1566, 1505, 1377, 1201, 1150, 1085 cm^{-1}; **^{1}H NMR** (300 MHz, $CDCl_3$) δ 8.48 (s, 1H), 8.23 (s, 1H), 7.95 (d, J = 9.0 Hz, 1H), 7.32 (d, J = 9.0 Hz, 1H), 3.43 (s, 3H), 3.39 (s, 3H), 2.33 (s, 3H), 2.32 (s, 3H); **^{13}C NMR** (151 MHz, $CDCl_3$) δ 168.6, 168.1, 162.9, 160.9, 157.4, 151.7, 146.4,

142.3, 133.8, 131.5, 129.4, 123.8, 118.5, 29.8, 29.1, 21.3, 21.2; **HRMS** (ESI-QTOF) m/z $[M+Na]^+$ calcd for $C_{17}H_{16}N_2O_7Na$ 383.0855, found 383.0855.

5-[(4-acetoxy-3-hydroxyphenyl)methylidene]-1,3-dimethyl-1,3-diazinane-2,4,6-trione (49)



Heterodiene **49** was obtained from compound **48**. Heterodiene **48** was synthesized by a Knoevenagel condensation of a commercially available aldehyde **42** and 1,3-dimethylbarbituric acid in water according to procedure described in literature.[34] The acetylation of compound **48** by Schoetten-Baumann method yielded only the monoacetyl derivative **49**. Compound **49** needed no further purification and was obtained with 90% yield. Compound **49**: white solid, mp 200-201 °C; **IR** (powder) ν 3235, 3089, 2954, 2930, 1724, 1664, 1543, 1506, 1421, 1299, 1226, 1156, 1081 cm^{-1}; **^{1}H NMR** (300 MHz, $CDCl_3$) δ 11.10 (br, 1H), 8.38 (d, *J* = 3.0 Hz, 1H), 8.25 (s, 1H), 8.00 (dd, *J* = 9.0, 3.0 Hz, 1H), 7.01 (d, *J* = 9.0 Hz, 1H), 3.21 (s, 6H), 2.30 (s, 3H); **^{13}C NMR** (151 MHz, $CDCl_3$) δ 163.9, 161.9, 157.9, 153.6, 152.2, 145.9, 137.4, 132.5, 125.4, 122.3, 116.5, 114.5, 29.7, 29.1, 21.6; **HRMS** (ESI-QTOF) m/z $[M+Na]^+$ calcd for $C_{15}H_{14}N_2O_6Na$ 341.0749, found 341.0750.

Stability studies of heterodienes and cycloaddition products

Stability studies of 5-arylidenebarbituric acids and cycloaddition products in different pH values

To analyze the stability of 5-arylidenebarbituric acids or cycloaddition products in different pH values, appropriate compounds (0.4 mmol) were added in to 20 mL of pH=4.0 or pH=7.4 buffer solution. The prepared mixtures were heated at 37 °C for 24 h. Extractions with dichloromethane (3x10 mL) were performed, the organic phases were dried over anhydrous magnesium sulfate and then evaporated. ^{1}H NMR spectra of the reaction mixture were recorded and compared to the spectra of pure heterodienes.

Stability studies of 5-arylidenebarbituric acids and cycloaddition products in the presence of cysteine or lysine

Aqueous solutions of heterodienes and amino acids (20 mL, 20 mM) were prepared. To 0.4 mmol of the heterodiene in 20 mL of water, respectively 48 mg (0.4 mmol) cysteine or 58 mg (0.4 mmol) lysine was added. The prepared solutions were heated at 37 °C for 24 h. Extractions with dichloromethane (3x10 mL) were performed, the organic phases were dried over anhydrous magnesium sulfate and then evaporated. ^{1}H NMR spectra of the reaction mixture were recorded and compared to the spectra of pure heterodienes.

Selected research results

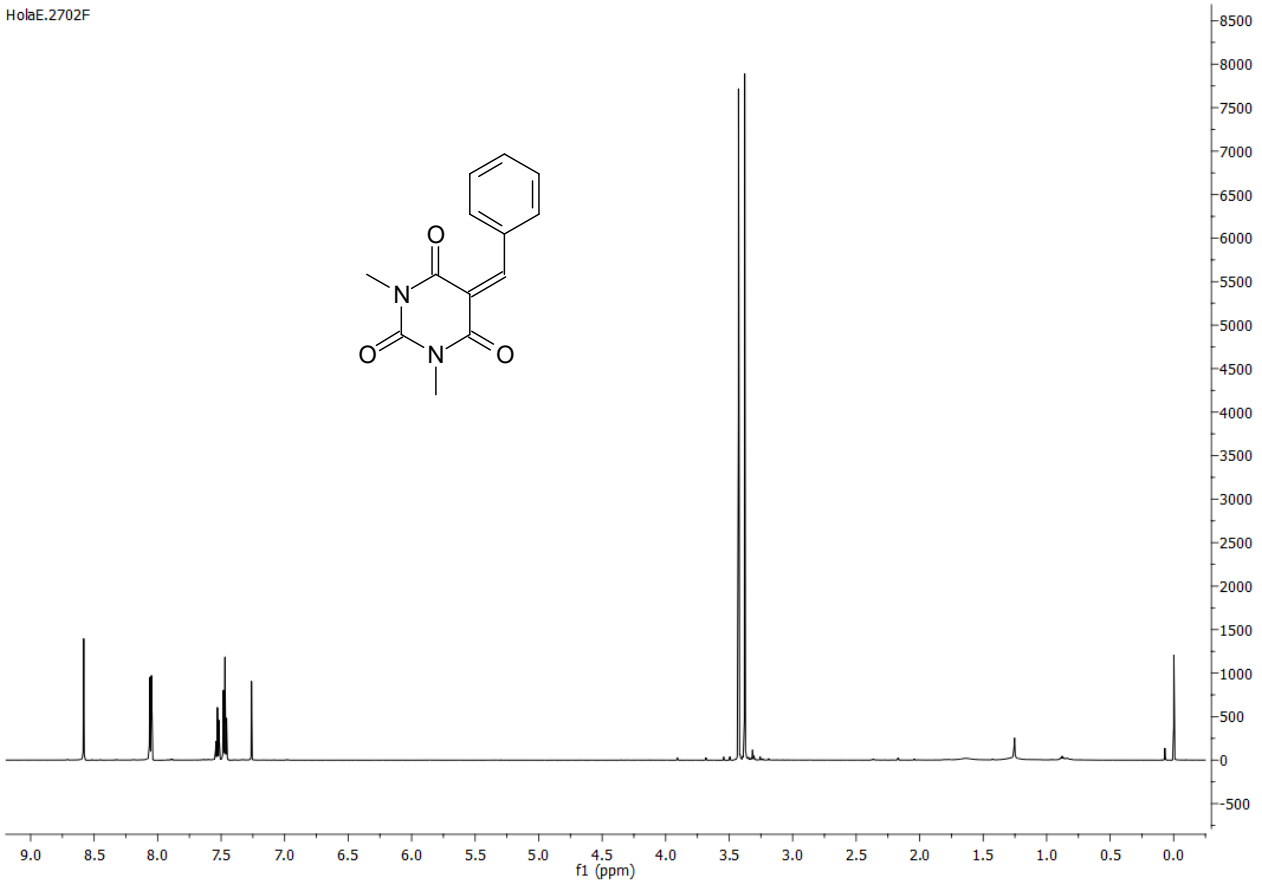


Fig. S1 Stability study of 5-phenylmethylidene-1,3-dimethyl-1,3-diazinane-2,4,6-trione in pH=4.0 buffer solution, 37 °C, 24h.

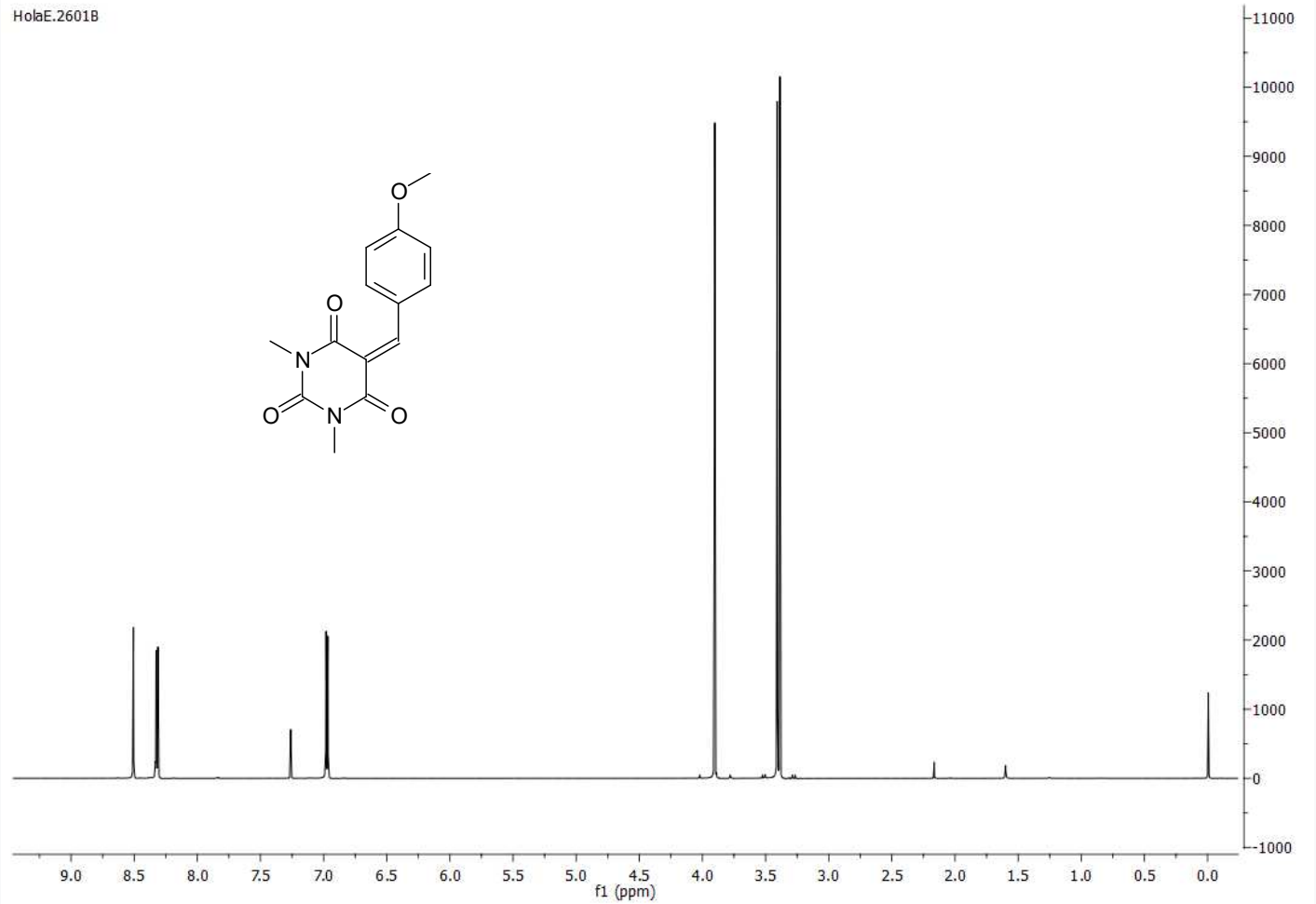


Fig. S2 Stability study of 5-[(4-methoxyphenyl)methylidene]-1,3-dimethyl-1,3-diazinane-2,4,6-trione -2,4,6-trione in pH=7.4 buffer solution, 37 °C, 24h.

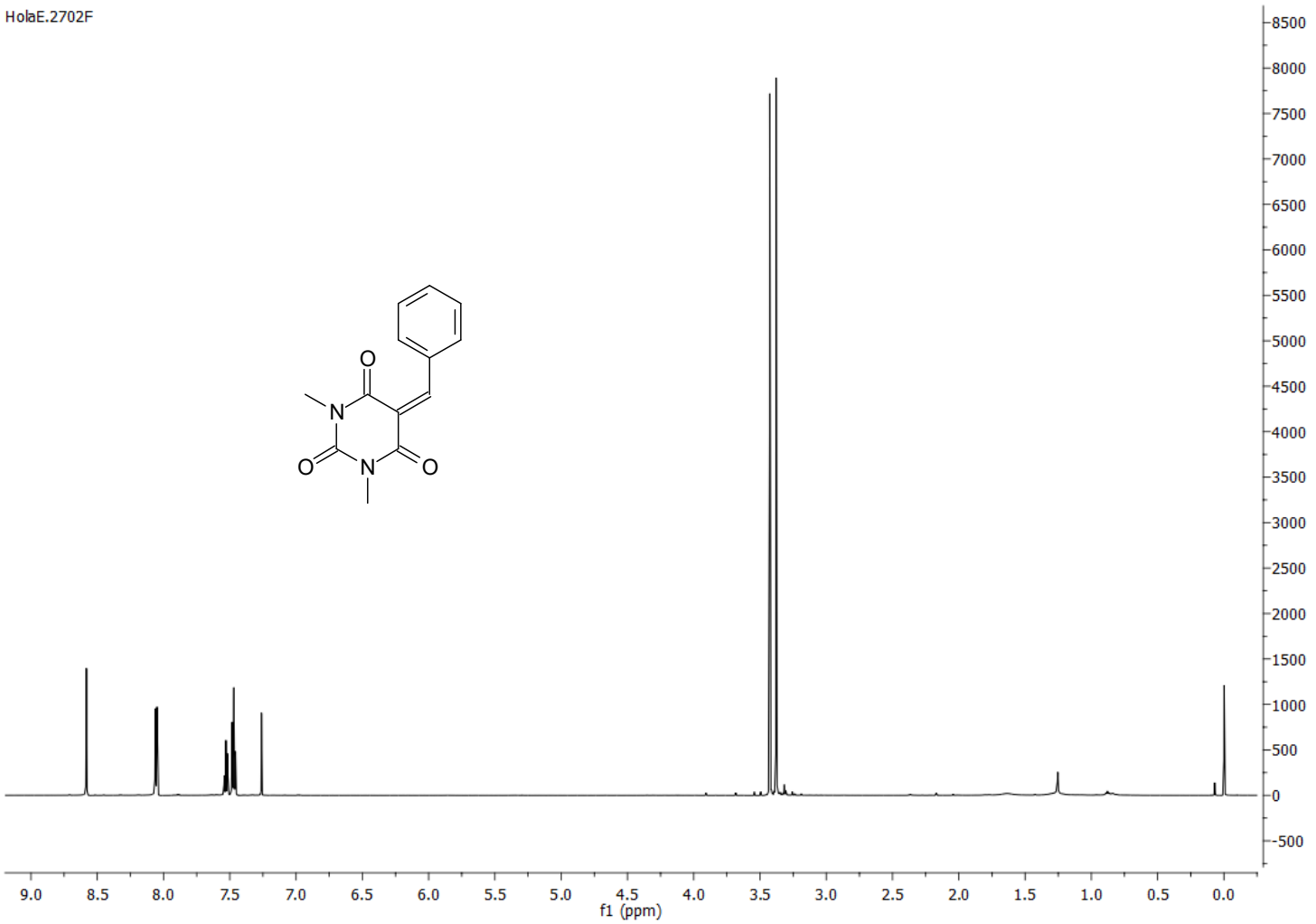


Fig. S3 Stability study of 5-phenylmethylidene-1,3-dimethyl-1,3-diazinane-2,4,6-trione in H_2O in the presence of lysine, 37 °C, 24h.

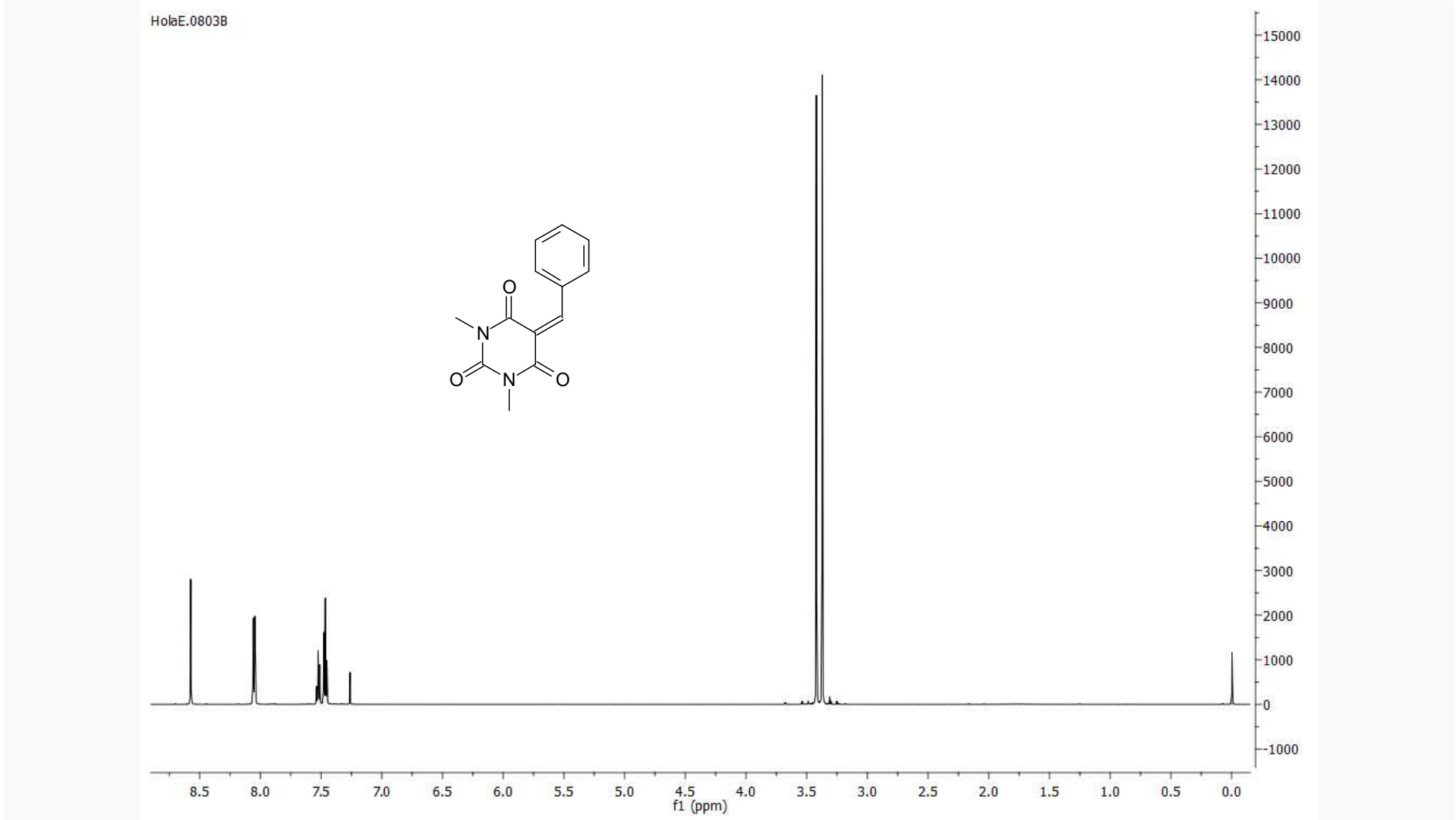


Fig. S4 Stability study of 5-phenylmethylidene-1,3-dimethyl-1,3-diazinane-2,4,6-trione in H_2O in the presence of cysteine, 37 °C, 24h.

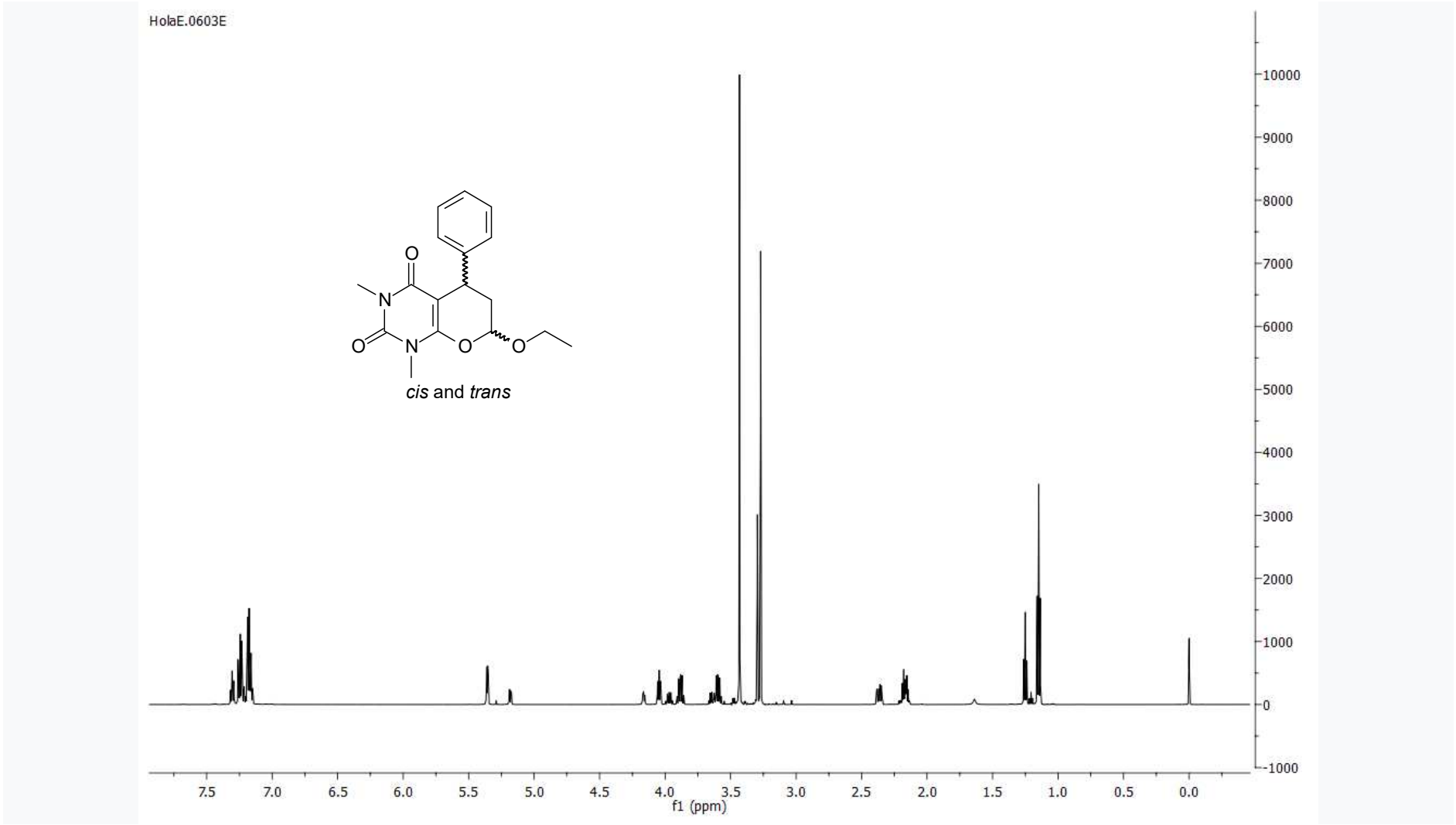


Fig. S5 Stability study of mixture of *cis* and *trans* diastereoisomers of 7-ethoxy-1,5,6,7-tetrahydro-1,3-dimethyl-5-phenyl-2*H*-pyrano[2,3-*d*]pyrimidine-2,4(3*H*)-dione in H_2O in the presence of lysine, 37 °C, 24h.

Stability studies of the cycloaddition product **39** in different pH values

To analyze the stability of cycloaddition product **39** in different pH values, **39** (0.0035 mmol) was dissolved in 1 mL of pH=4.0 and pH=7.4 buffer solution at 37 °C. The resulting solutions were monitored by LC-MS after 24 h.

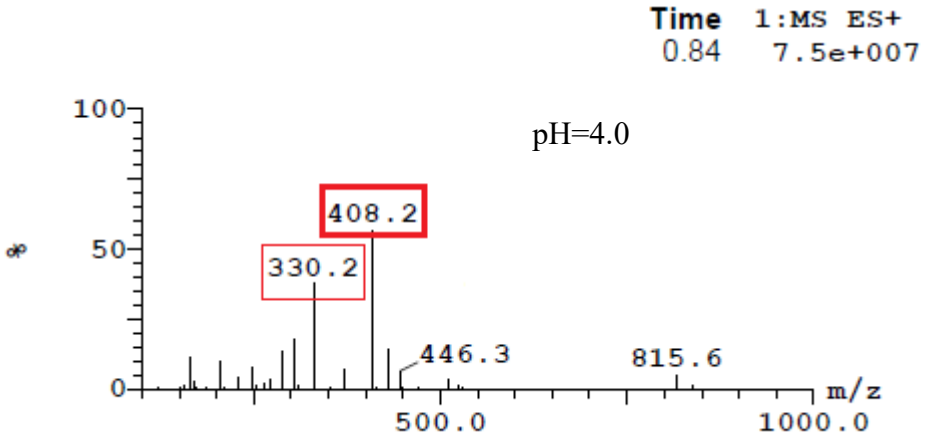


Fig. S6 Stability studies of the cycloaddition product **39** in pH=4.0 buffer solution.

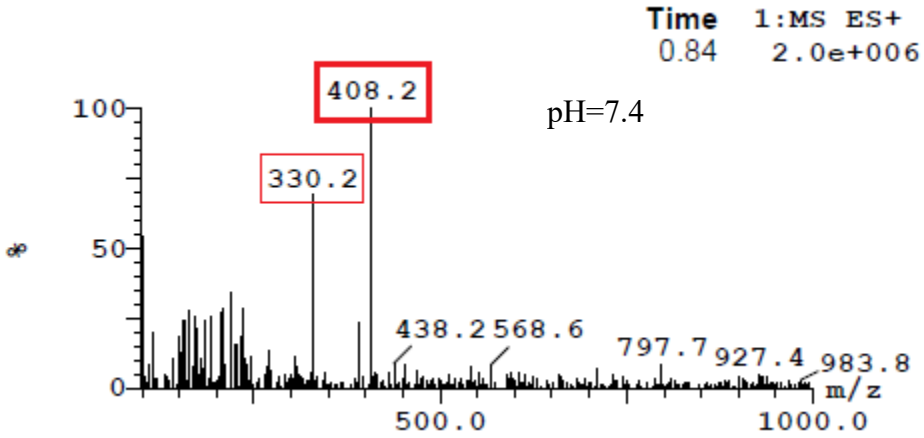


Fig. S7 Stability studies of the cycloaddition product **39** in pH=7.4 buffer solution.

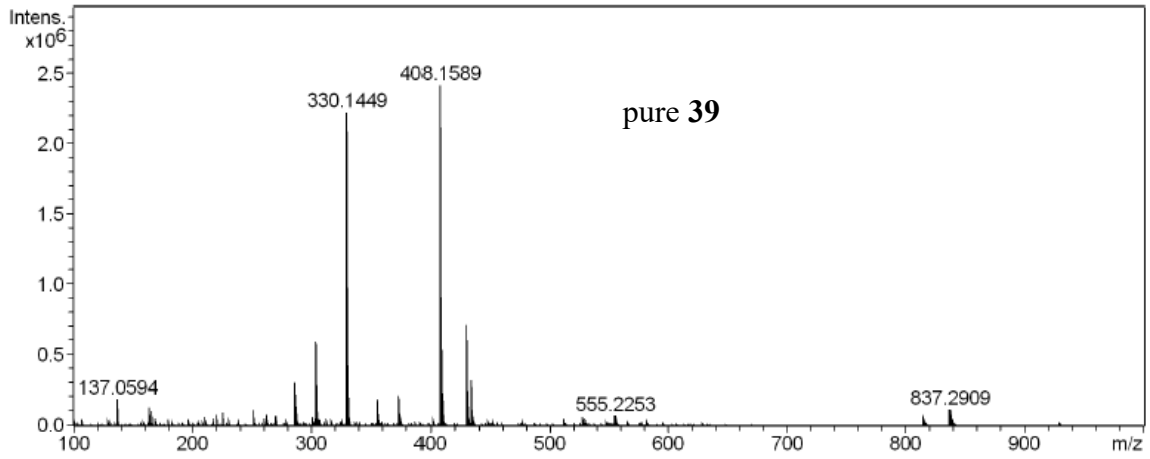
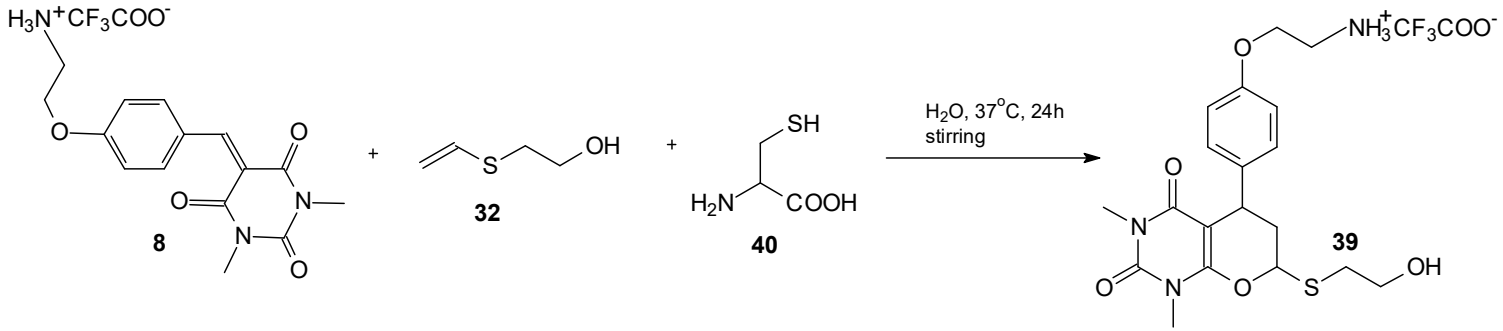


Fig. S8. HRMS (ESI-QTOF) of cycloadduct **39,** m/z $[M\text{-}CF_3COO]^+$ calcd for $C_{19}H_{26}N_3O_5S$ 408.1593, found 408.1589.

It was found that **39** is stable at pH 4.0−7.4 (Fig. S8 and Fig. S9; mass peaks characteristic for the product **39** are marked with red boxes, bold red box – main peak).

Studies of cycloaddition reaction of **8** and **32** in the presence of cysteine **40**

Heterodiene **8** (0.1 mM) and dienophile **32** (1 mM) were treated with cysteine (0.1 mM) in H_2O at 37 °C, and the resulting solution was monitored via LC-MS after 24 h.



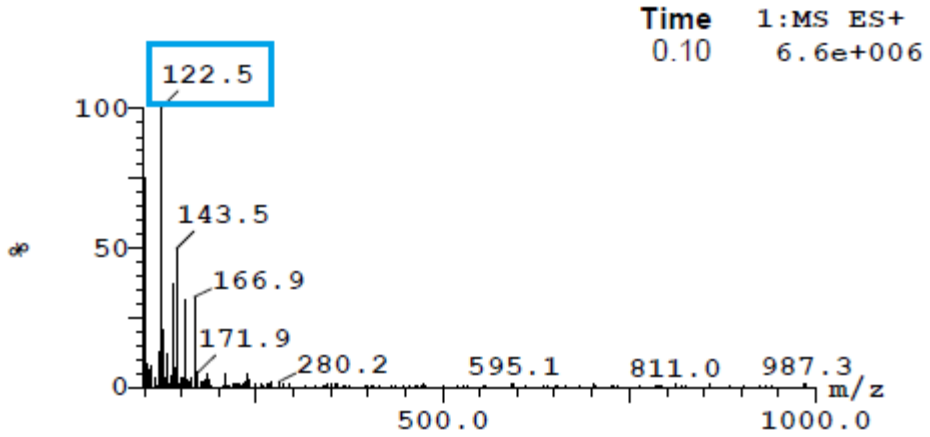


Fig. S9 Studies of cycloaddition reaction of **8** and **32** in the presence of cysteine **40**.

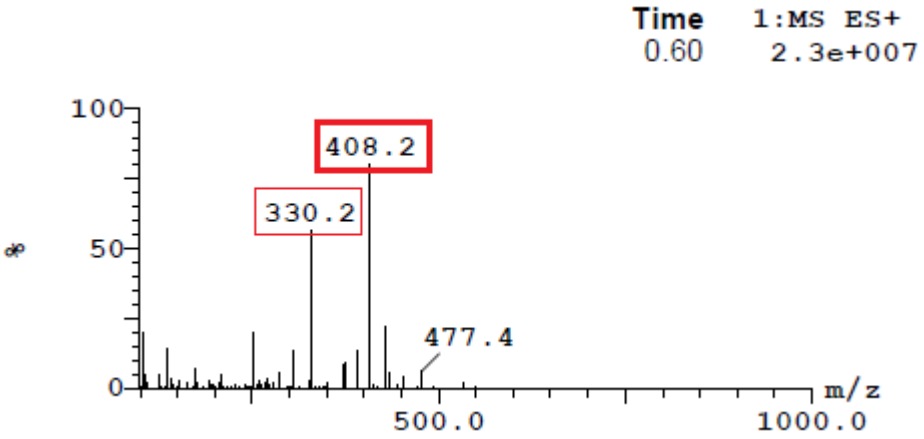
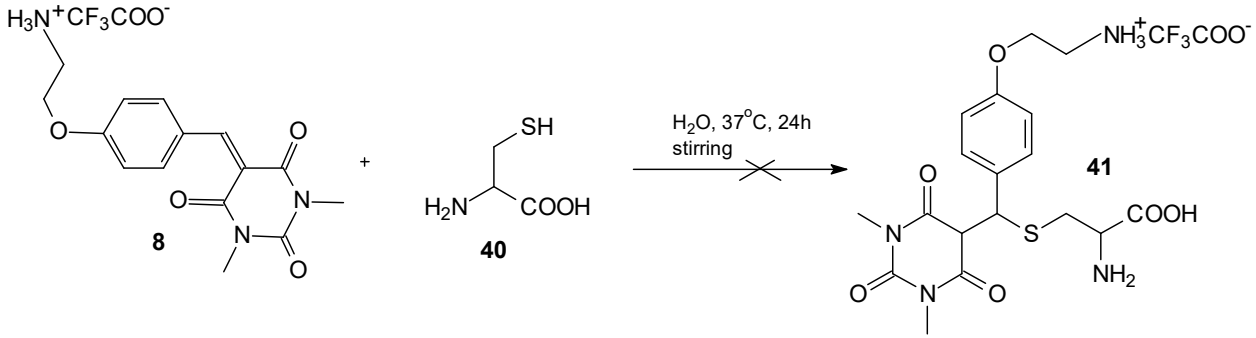


Fig. S10 Studies of cycloaddition reaction of **8** and **32** in the presence of cysteine **40**.

The reaction of heterodiene **8** and dienophile **32** in the presence of cysteine **40** was investigated (Fig. S9 and Fig. S12). The HDA cycloadduct **39** (mass peaks characteristic for this compound are marked with red boxes) and also unreacted cysteine **40** (mass peak characteristic for this compound is marked with blue box) were observed in H_2O at 37 °C after 24 h.

Experiment concerning thiol Michael addition heterodiene **8** and cysteine **40**

A mixture of heterodiene **8** (0.01 mmol) and cysteine **40** (0.011 mmol) in 3 mL of H_2O was stirred at 37 °C, and the resulting solution was monitored via LC-MS after 24 h.



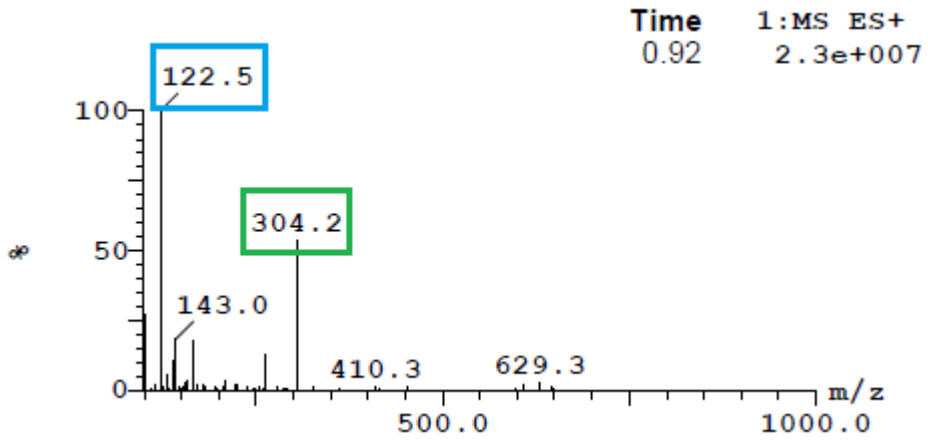


Fig. S11 Experiment concerning thiol Michael addition heterodiene **8** and cysteine **40**.

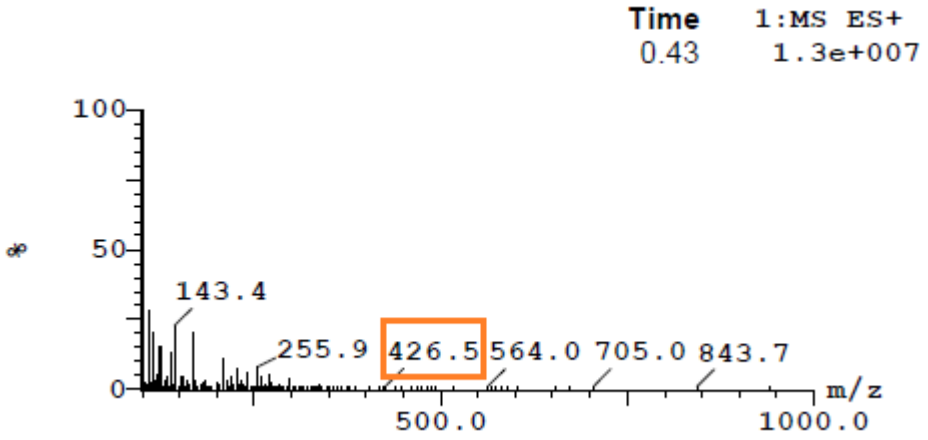


Fig. S12 Experiment concerning thiol Michael addition heterodiene **8** and cysteine **40**.

A reaction of heterodiene **8** and cysteine **40** was carried out to check the reactivity between these two compounds (Fig. S11 and Fig. S12). The intense mass peaks characteristic for both substrates **8** and **40** were observed after 24 h (peak for cysteine **40** in blue box, peak for heterodiene **8** in green box, Fig. S11). There was also a very small peak for thiol Michael addition product **41** (marked with orange box, Fig. S12).

Cycloaddition reactions rate study

The cycloaddition reaction of **8** and **32** rate study (indirect method)

^{1}H NMR was used to calculate the reaction rate constant of the hetero-Diels–Alder cycloaddition of heterodiene **8** and ether **32**. Heterodiene **8** (0.17 mmol) and ether **32** (1.7 mmol) were first dissolved in H_2O (50 mL), and the reaction mixture was stirred at 37 °C. A sample of 8 mL solution of the reaction mixture was collected every 20~30 min, evaporated under high vacuum and was analyzed by ^{1}H NMR in DMSO-d_6. Three samples were used to calculate the reaction rate constant after 80 min. Percent conversion was monitored by both disappearance of starting material and appearance of product as determined by integration at multiple chemical shifts. Pseudo-first order rate constant (k_{obs}) for the reaction was determined by plotting the ln [1/(1-x)] versus time and analysis by linear regression, x represents the percent conversion of compound **8**. The reaction was repeated in triplicate. The three lines had an average of $k_{obs} = 5.4333 \times 10^{-4}$ s^{-1}. Second order rate constant was estimated by k_{obs}/[**32**]. As a result, the second order rate constant k_2 for the cycloaddition of **8** and **32** is $(1.6 \pm 0.1) \times 10^{-2}$ M^{-1} s^{-1} in H_2O.

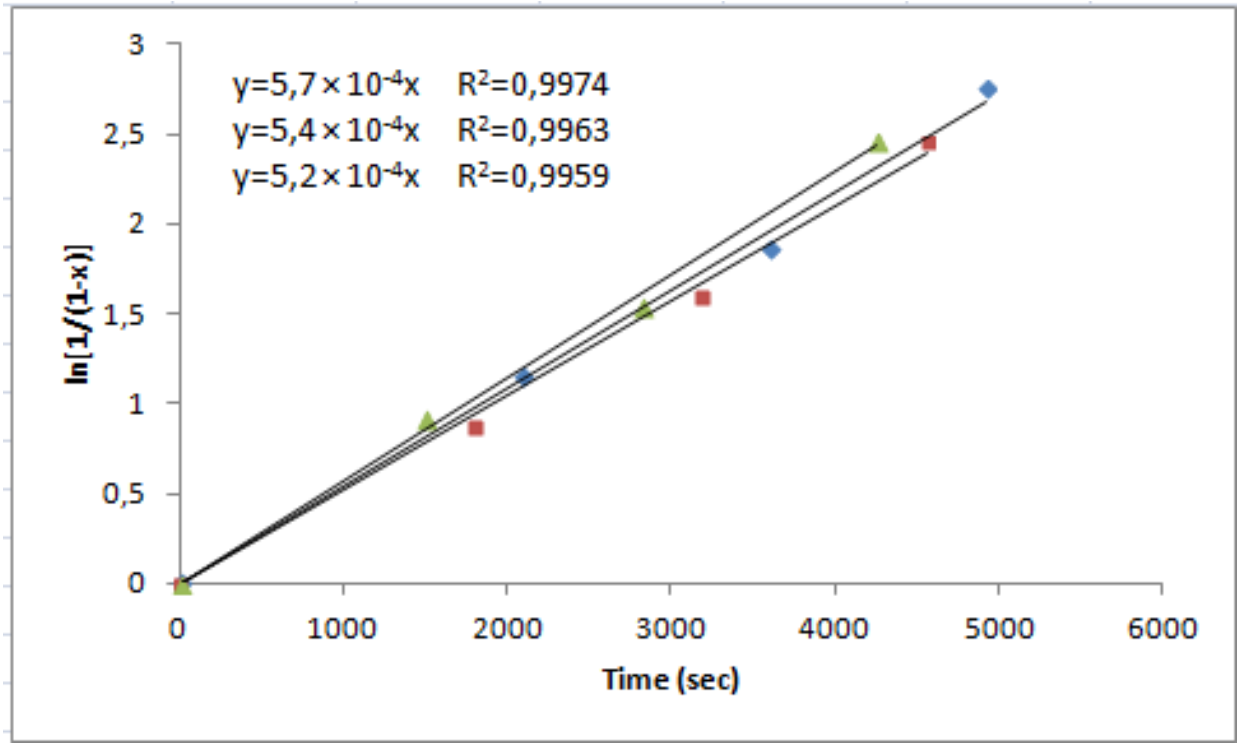
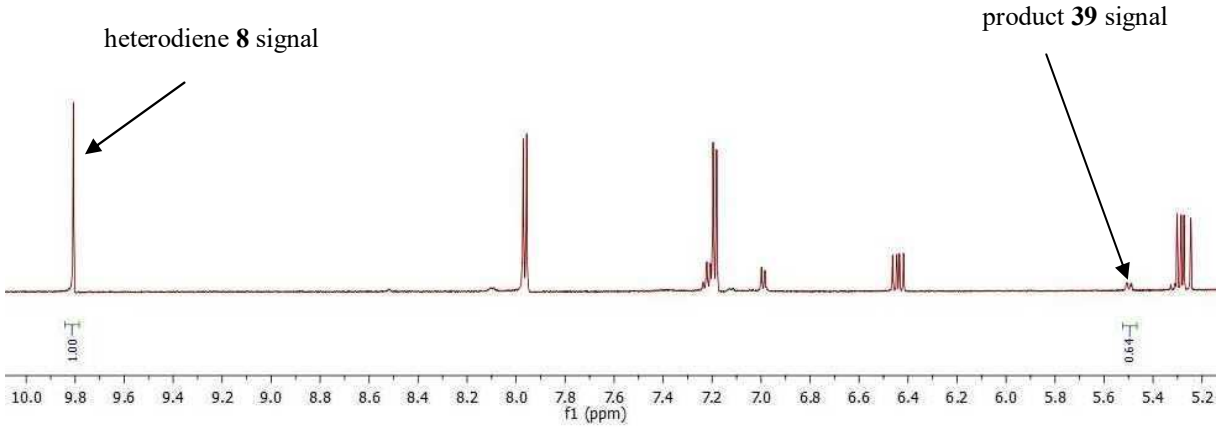


Fig. S13 The cycloaddition reaction of **8** and **32** rate study (indirect method).

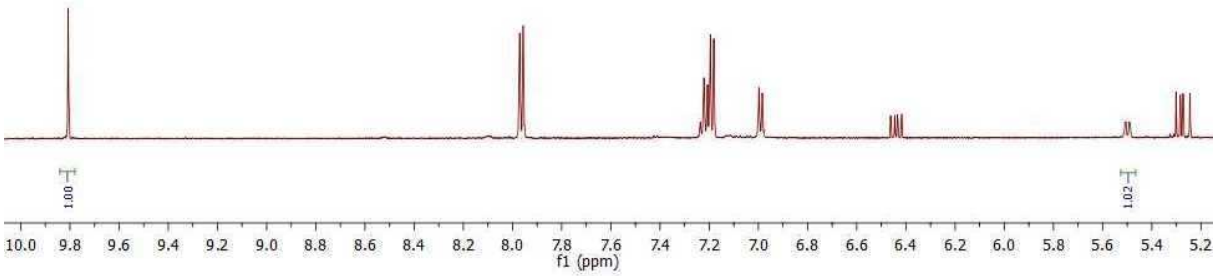
The cycloaddition reaction of **8** and **32** rate study (direct method)

^{1}H NMR was used to calculate the reaction rate constant of the hetero-Diels–Alder cycloaddition of heterodiene **8** and ether **32**. The reaction was carried out directly in the cuvette. Heterodiene **8** (0.0095 mmol) was first dissolved in D_2O (0.6 mL) and then added to solution of **32** (0.0095 mmol) in D_2O (0.2 mL). The temperature in the spectrometer was 27 °C. Spectra were collected every 7 min. 3 spectra were chosen and used to calculate the reaction rate constant. Percent conversion was monitored by both disappearance of starting material (heterodyne **8**) and appearance of product **39** as determined by integration at multiple chemical shifts. The reaction was repeated in triplicate. The key signals used to determine the disappearance of starting material and appearance of product are shown in the following spectra:

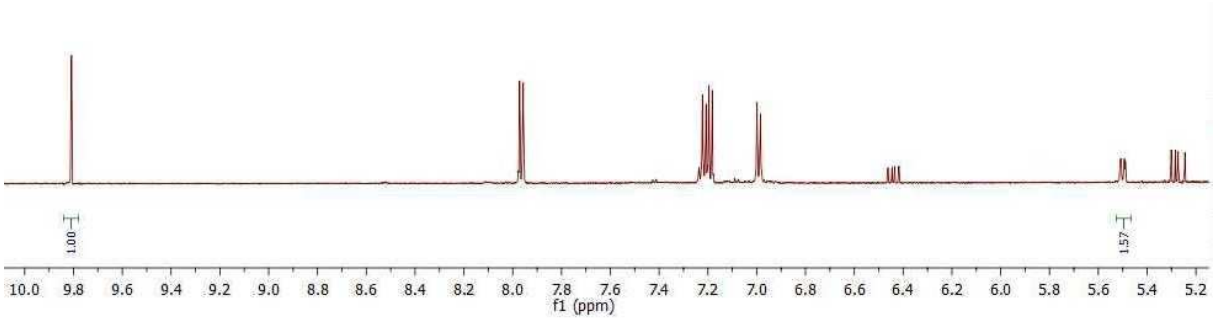
time: 49 min, x = 61% (% of substrate remaining in the sample)



time: 98 min, x = 49,5%



time: 154 min, x = 38,9%



Second order rate constant k_2 for the reaction **8** and **32** was determined by plotting the $1/c_D$ - $1/c_{D,0}$ versus time and analysis by linear regression. $c_{D,0}$ represents the initial concentration of diene and c_D represents the concentration of the diene at the appropriate measuring point. c_D was calculated based on $c_{D,0}$ and x (% of substrate remaining in the sample). A slope factor (a) is equal to the second order rate constant k_2, in accordance with second order rate kinetics. The three lines had an average of slope factor a = 1.4×10^{-2}. As a result, the second order rate constant for the cycloaddition of **8** and **32** is $(1.4 \pm 0.1) \times 10^{-2}$ M^{-1} s^{-1} in D_2O.

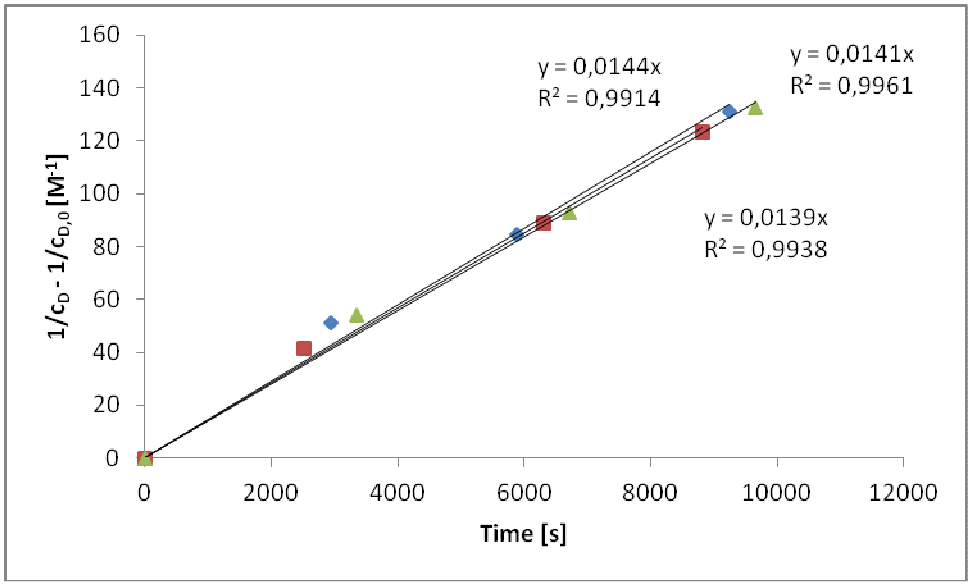


Fig. S14 The cycloaddition reaction of **8** and **32** rate study (direct method).

The cycloaddition reaction of **9** and **32** rate study

^{1}H NMR was used to calculate the reaction rate constant of the hetero-Diels-Alder cycloaddition of hetrodiene **9** and ether **32**. Hetrodiene **9** (0.225 mmol) and ether **32** (2.25 mmol) were first dissolved in H_2O (50 mL), and the reaction mixture was stirred at 37 °C. A sample of 8 mL solution of the reaction mixture was collected every 20~30 min, evaporated under high vacuum and was analyzed by ^{1}H NMR in DMSO-d_6.

Three samples were used to calculate the reaction rate constant after 80 min. Percent conversion was monitored by both disappearance of starting material and appearance of product as determined by integration at multiple chemical shifts. Pseudo-first order rate constant (k_{obs}) for the reaction was determined by plotting the ln [1/(1-x)] versus time and analysis by linear regression, x represents the percent conversion of compound **17**. The reaction was repeated in triplicate. The three lines had an average of $k_{obs} = 4.0667 \times 10^{-4}$ s^{-1}. Second order rate constant was estimated by k_{obs}/[**32**]. As a result, the second order rate constant for the cycloaddition of **9** and **32** is $(0.9\pm 0.1) \times 10^{-2}$ M^{-1} s^{-1} in H_2O.

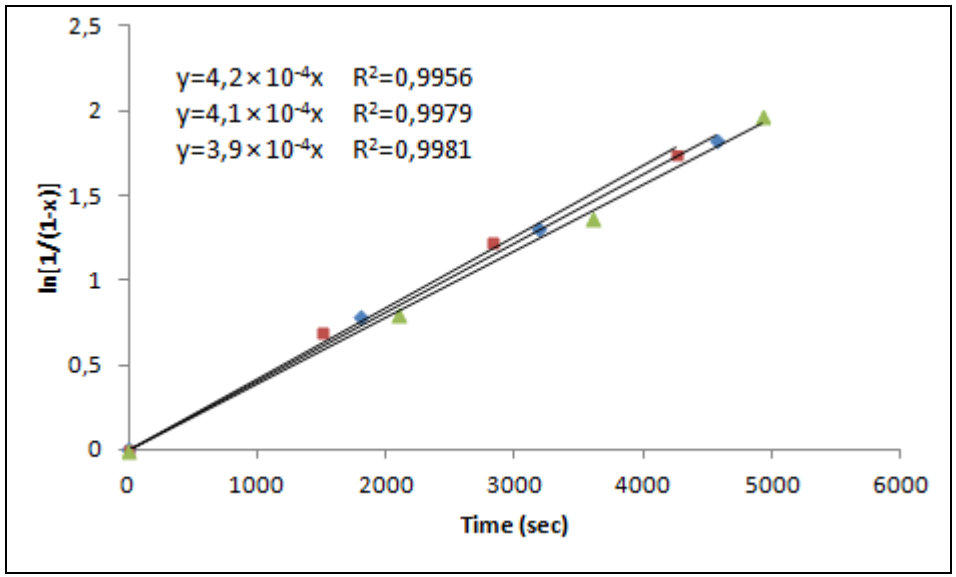


Fig. S15 The cycloaddition reaction of **9** and **32** rate study.

Rate study of cycloaddition reaction of **44** and **32** and reaction of **46** and **32**

An attempt was made to determine the rate constant k_2 of reaction of compounds **44** and **32**. First, the same measurement conditions were applied as for the studies of the kinetics of the reaction of compounds **8** and **32** or **9** and **32**. However, measurement of the first ^{1}H NMR spectrum of the reaction mixture indicated that the reaction was complete because no signals for the heterodiene **44** were visible in the spectrum (Fig. S16). The same result was obtained for the reaction of the monoacetyl derivative **46** and ether **32**. Only cycloadduct **47** signals were visible in the measured spectrum (Fig. S17).

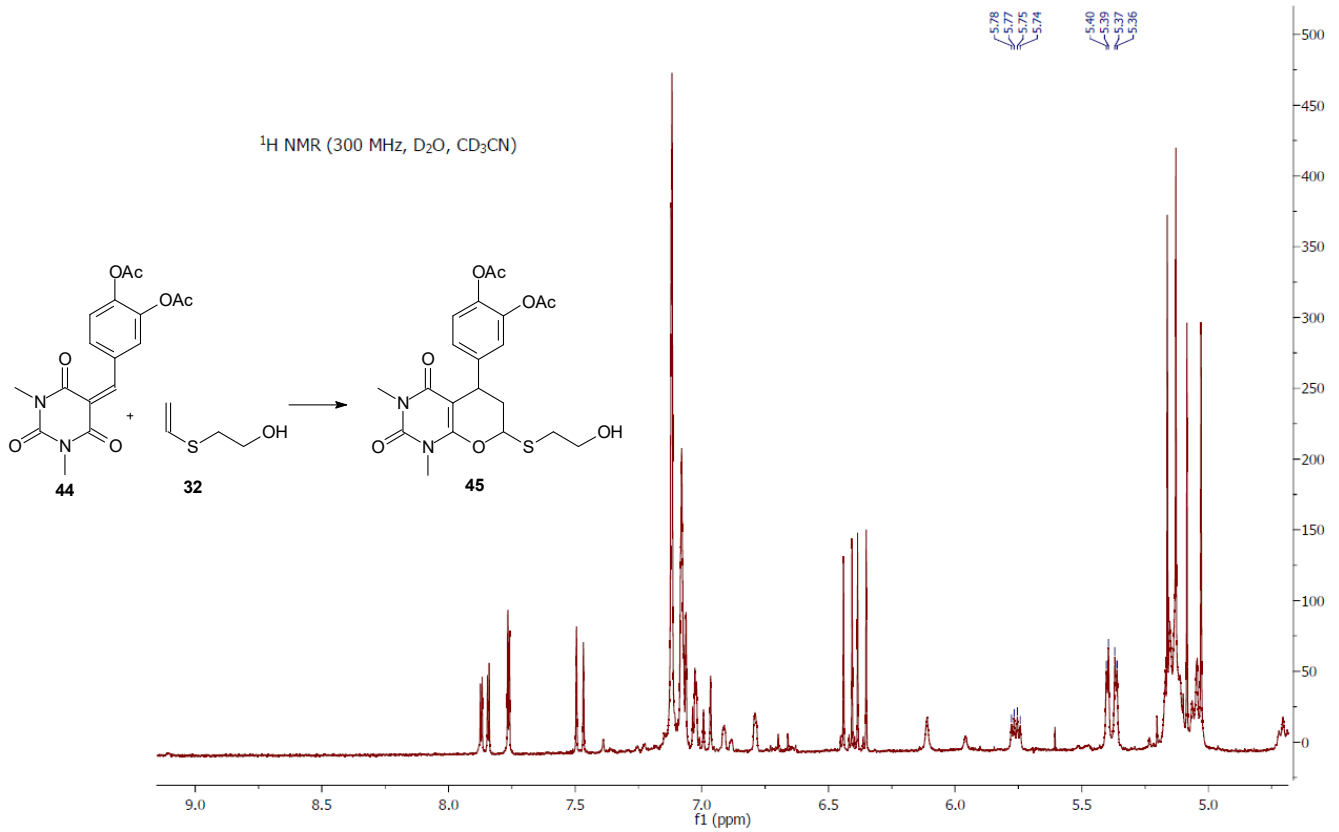


Fig. S16 ^{1}H NMR spectrum of the first sample taken from the reaction mixture of heterodiene **44** and vinyl thioether **32**.

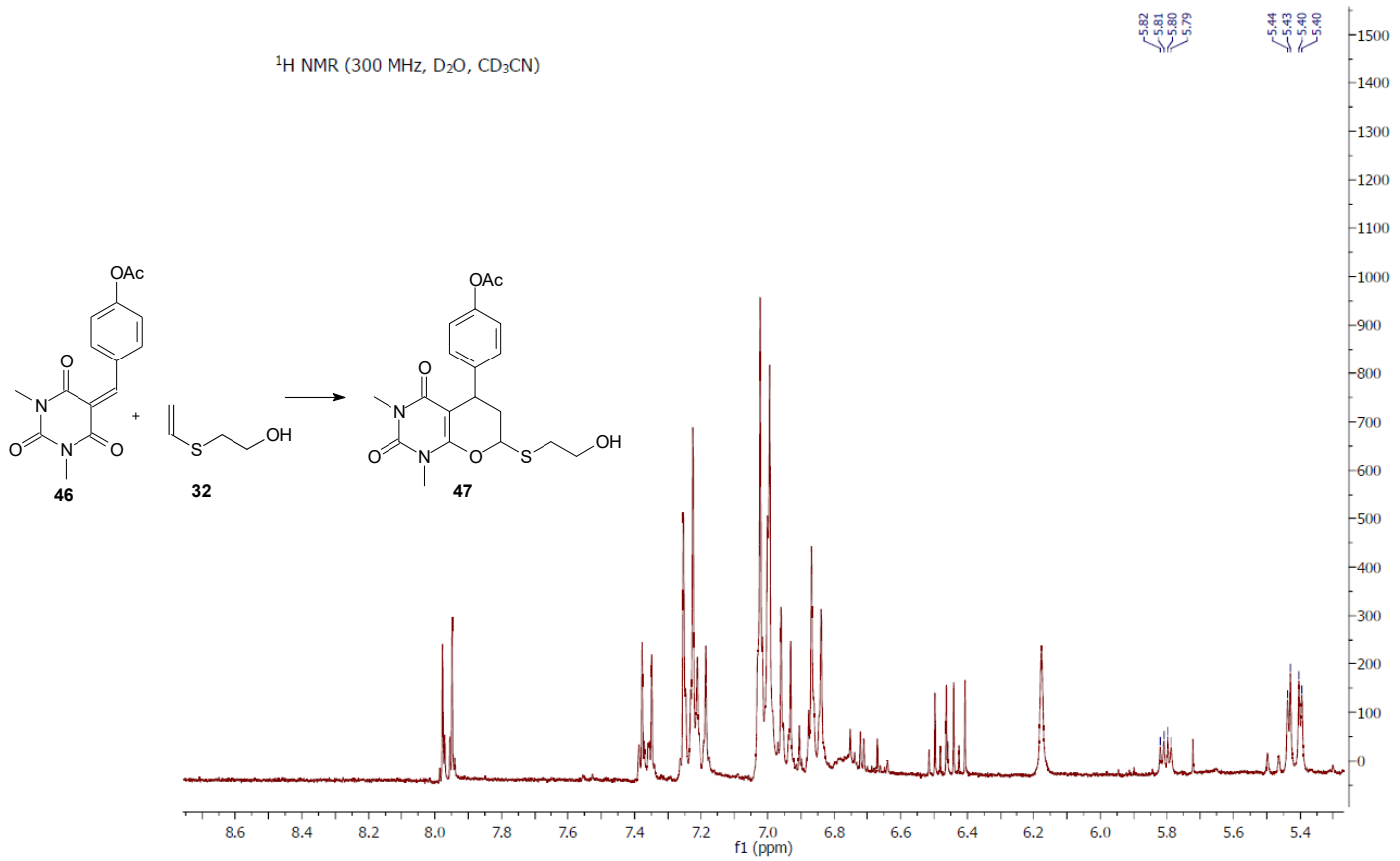
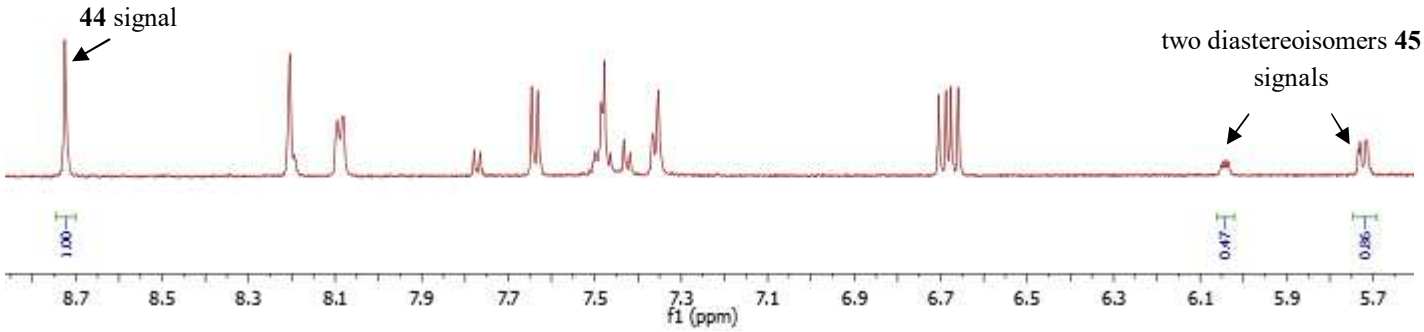


Fig. S17 ^{1}H NMR spectrum of the first sample taken from the reaction mixture of heterodiene **46** and vinyl thioether **32**.

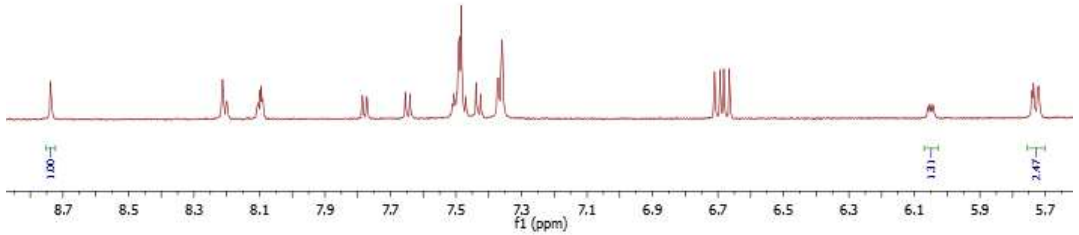
The cycloaddition reaction of **44** and **32** rate study by the direct method in the NMR cuvette, the molar ratio of the reactants 1:1, D_2O/CD_3CN (3:1)

^{1}H NMR was used to calculate the reaction rate constant k_2 of the hetero-Diels–Alder cycloaddition of **44** and **32**. The reaction was carried out directly in the NMR cuvette. **44** (4.5 mg, 0.0125 mmol) was first dissolved in CD_3CN (0.2 mL) and then added to solution of **32** (1,3 mg, 0.0125 mmol) in D_2O (0.6 mL). The temperature in the spectrometer was 27 °C. Spectra were collected every 27 s. 5 spectra were chosen and used to calculate the reaction rate constant k_2. Percent conversion was monitored by both disappearance of starting material **44** and appearance of products (two diasteroisomers of **45**) as determined by integration at multiple chemical shifts. The reaction was repeated in triplicate. The key signals used to determine the disappearance of starting material and appearance of products are shown in the following spectra:

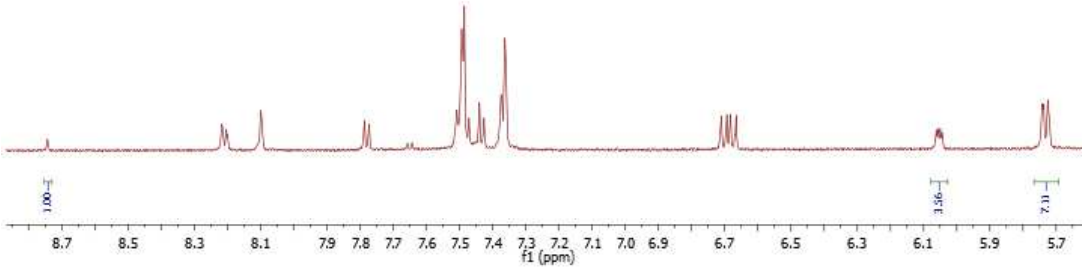
time: 81 s, x = 43% (% of substrate **44** remaining in the sample)



time: 189 s, x = 21%



time: 486 s, x = 8,6%



Second order rate constant k_2 for the reaction **44** and **32** was determined by plotting the $1/c_D$ - $1/c_{D,0}$ versus time and analysis by linear regression. $C_{D,0}$ represents the initial concentration of heterodiene **44** and c_D represents the concentration of the heterodiene **44** at the appropriate measuring point. c_D was calculated based on $c_{D,0}$ and x (% of substrate remaining in the sample). A slope factor (a) is equal to the second order rate constant

(k_2), in accordance with second order rate kinetics. The three lines had an average of slope factor a = 1.4. As a result, the second order rate constant k_2 for the cycloaddition of **44** and **32** is 1.4± 0.1 M^{-1} s^{-1} in D_2O/CD_3CN (3:1).

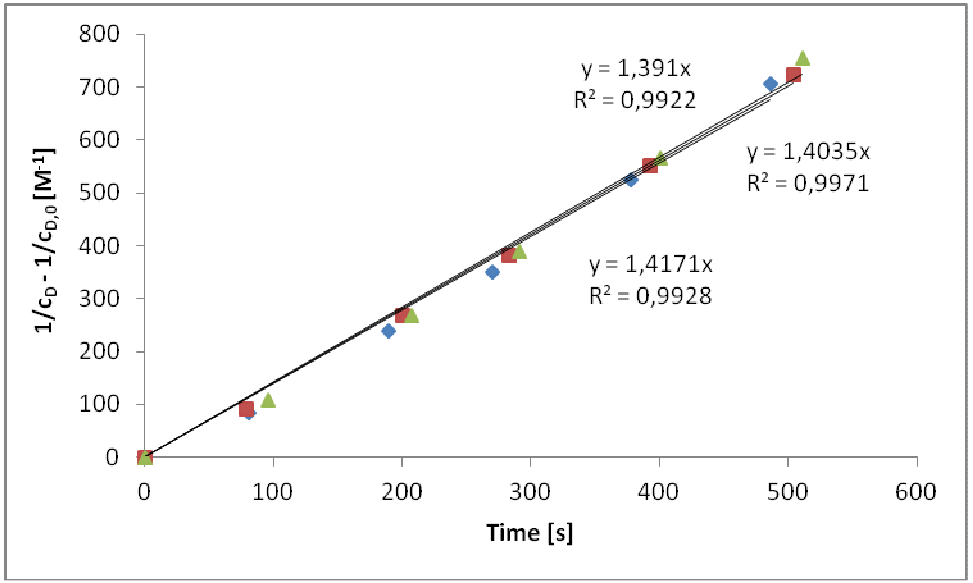
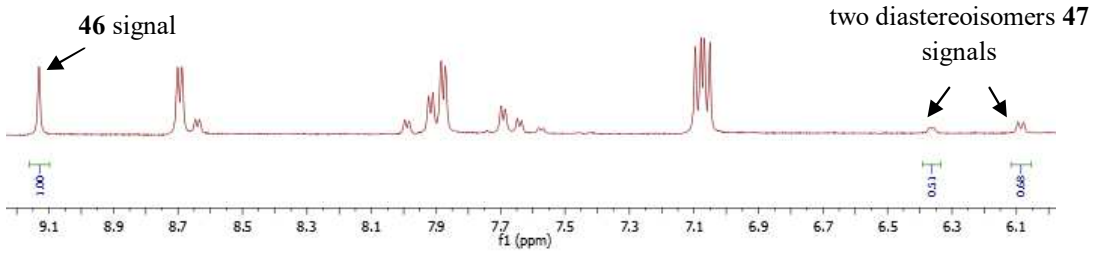


Fig. S18 The cycloaddition reaction of **44** and **32** rate study by the direct method in the NMR cuvette, the molar ratio of the reactants 1:1, D_2O/CD_3CN (3:1).

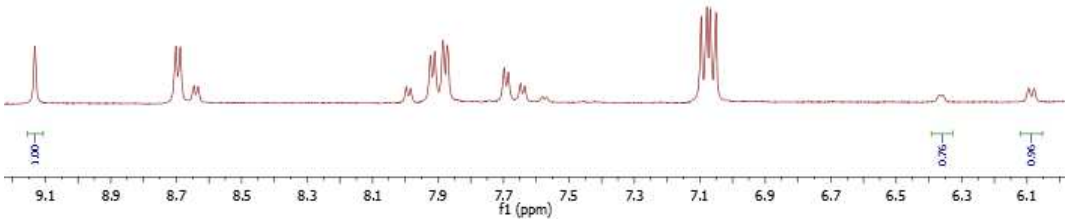
The cycloaddition of **46** and **32** rate study by the direct method in the NMR cuvette, the molar ratio of the reactants 1:1, D_2O/CD_3CN (3:5)

^1H NMR was used to calculate the reaction rate constant k_2 of the hetero-Diels–Alder cycloaddition of **46** and **32**. The reaction was carried out directly in the NMR cuvette. **46** (2.25 mg, 0.00745 mmol) was first dissolved in CD_3CN (0.5 mL) and then added to solution of **32** (3 mg, 0.02885 mmol) in D_2O (0.3 mL). The temperature in the spectrometer was 27 °C. Spectra were collected every 27 s. 4 spectra were chosen and used to calculate the reaction rate constant k_2. Percent conversion was monitored by both disappearance of starting material **46** and appearance of products (two diastereoisomers of **47**) as determined by integration at multiple chemical shifts. The reaction was repeated in triplicate. The key signals used to determine the disappearance of starting material and appearance of products are shown in the following spectra:

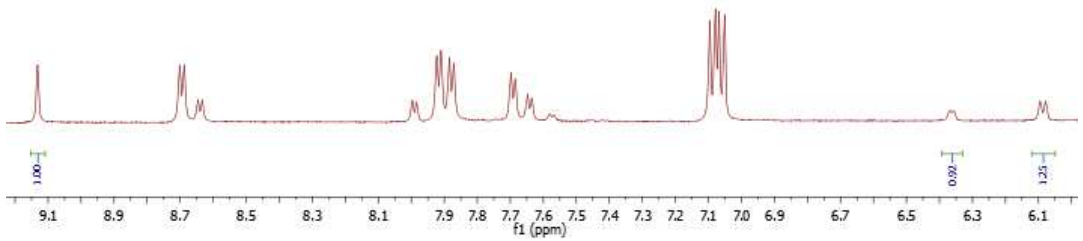
time: 317 s, x = 46% (% of substrate remaining in the sample)



time: 441 s, x = 37%



time: 546 s, x = 31,5%



Second order rate constant k_2 for the reaction **46** and **32** was determined by plotting the $1/c_D$ - $1/c_{D,0}$ versus time and analysis by linear regression. $C_{D,0}$ represents the initial concentration of diene **46** and c_D represents the concentration of the diene **46** at the appropriate measuring point. c_D was calculated based on $c_{D,0}$ and x (% of substrate remaining in the sample). A slope factor (a) is equal to the second order rate constant (k_2), in accordance with second order rate kinetics. The three lines had an average of slope factor a = 4.3×10^{-1}. As a result, the second order rate constant k_2 for the cycloaddition of **46** and **32** is 0.43± 0.1 M^{-1} s^{-1} in D_2O/CD_3CN (3:5).

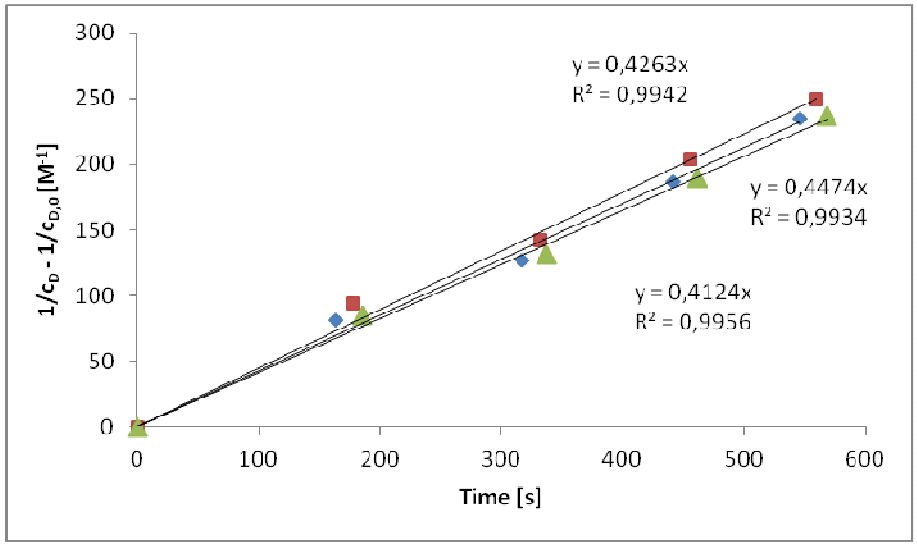


Fig. S19 The cycloaddition reaction of **46** and **32** rate study by the direct method in the NMR cuvette, the molar ratio of the reactants 1:1, D_2O/CD_3CN (3:5).

Bioorthogonality of the proposed cycloadditions

Cell culture

Human osteosarcoma cell line U-2 OS (ECACC, cat. no 92022711, lot no. 10K035) and human cervix epitheloid carcinoma cell line HeLa (ECACC, cat. no 93021013) were cultured in Mc Coy's medium (BioWest) supplemented with 10% Foetal Bovine Serum (FBS, BioWest). The cells were grown at 37°C in a humified atmosphere containing 5% CO_2.

Cell viability MTT assay and dose-response curve fitting

HeLa and U-2 OS cells were seeded on 96-well transparent plates (Falcon) at the density of 5000 per well. The next day, the cells treated with the increasing concentrations of the indicated compounds, prepared as 1000x stock solutions in DMSO. Also, DMSO was used as a control. The cells were treated for 48 hours and then Thiazolyl Blue Tetrazolium Bromide (MTT, Sigma Aldrich) was added for 30 minutes at a final concentration of 0.5 mg/ml. The medium was carefully removed and MTT crystals were dissolved in isopropanol supplemented with 40 mM HCl. The absorbance was measured with the Spark microplate reader (Tecan Group Ltd.) at 570 nm with the reference wavelength 650 nm for background subtraction. For data analysis, a dose-response fitting was performed using the Origin 2020 software.

Fluorescence labeling studies

U-2 OS or HeLa cells were plated on Cell Culture Slides (Biologix, cat. 07-2104) at a density of 40 000 cells/cm^2 in the culture medium. The next day, the cells were treated with DMSO or dienophile-taxol **38** (20 μM for U-2 OS and 0.5 μM for HeLa) at 37 °C for 2 h and then washed three times with PBS. The cells were then treated with FITC (100 μM) or heterodiene-fluorescein **30** (33.3 μM for U-2 OS and 20 μM for HeLa) for additional 10 hours at the culture conditions, washed three times with PBS and submerged with fresh PBS containing Hoechst 33342 for the microscopy analysis performed with the use of Leica DM IL Led fluorescence microscope, equipped with Leica DFC3000 G digital camera. The images were captured using Leica Application Siute X 3.3.3.16958 software and analysed with ImageJ 1.52i software.[37]

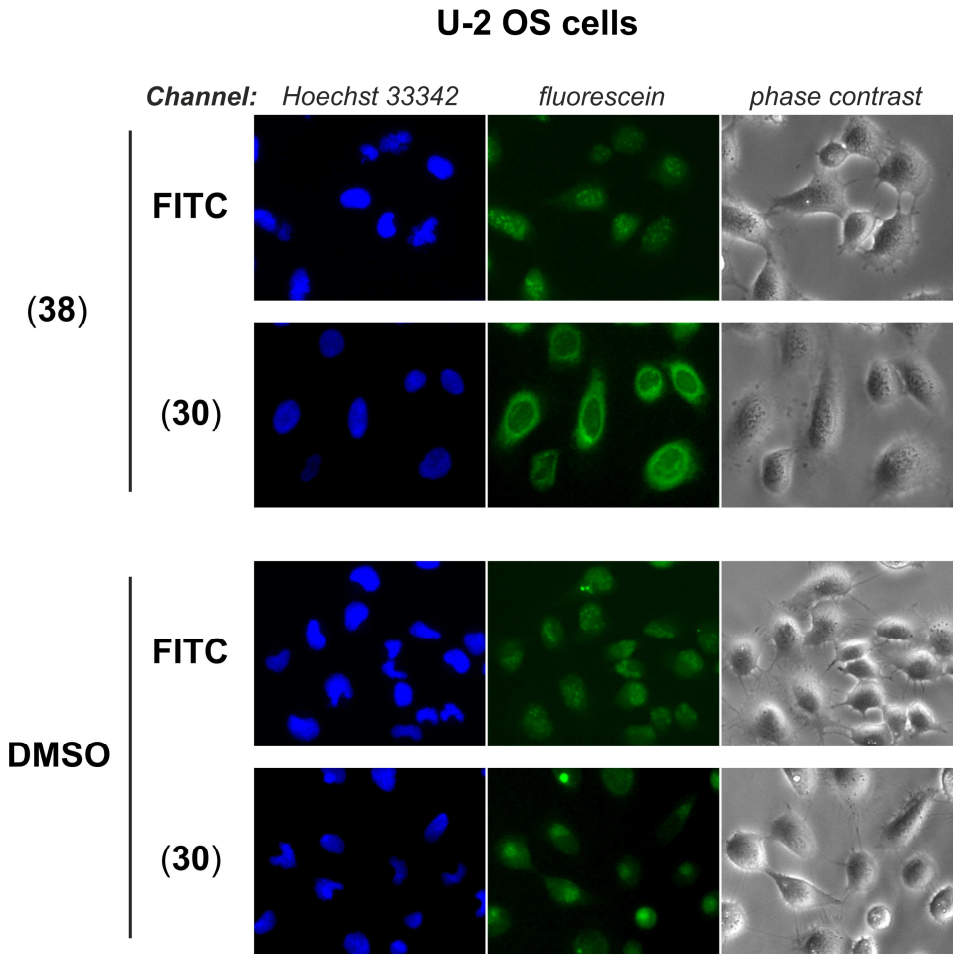
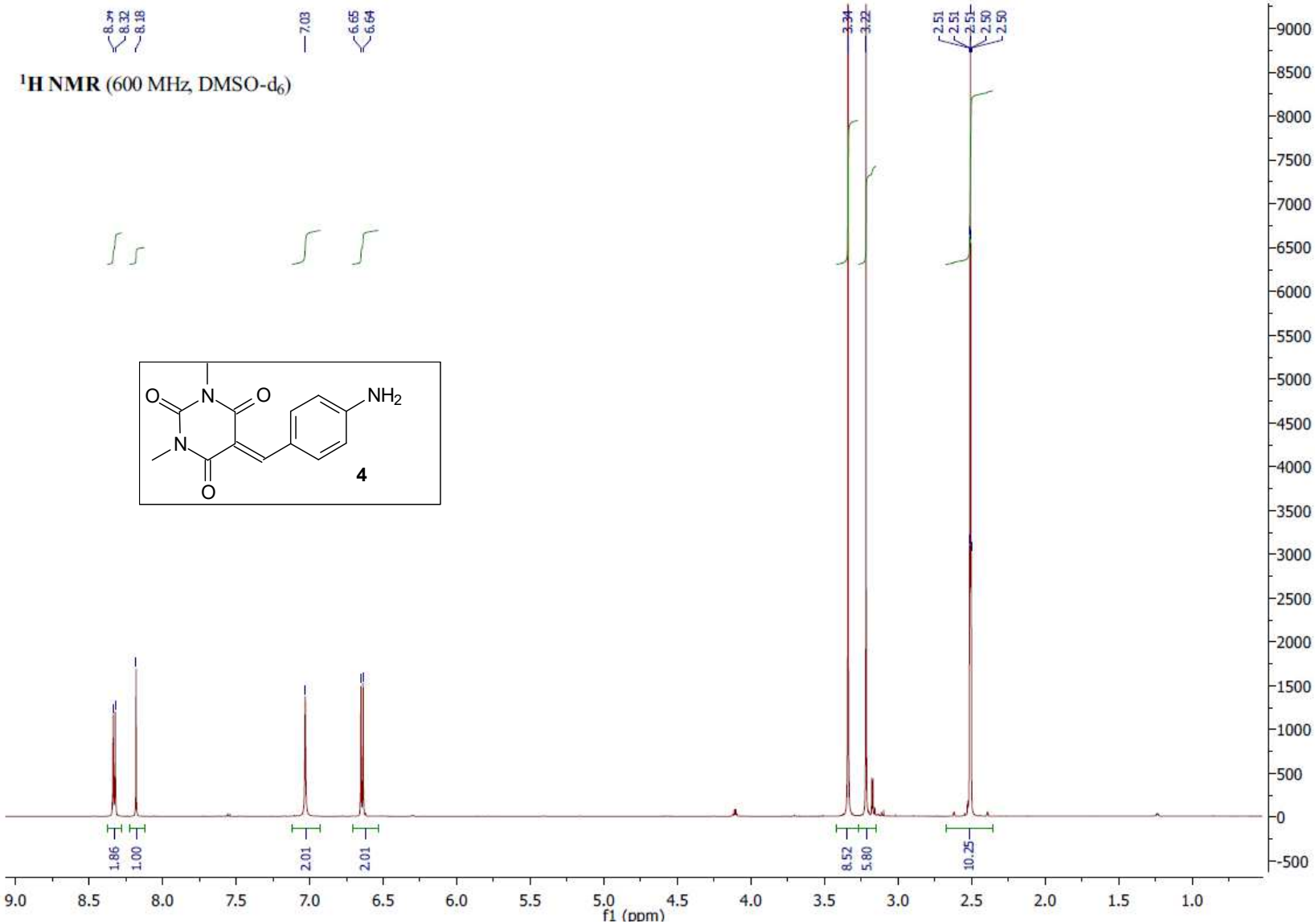
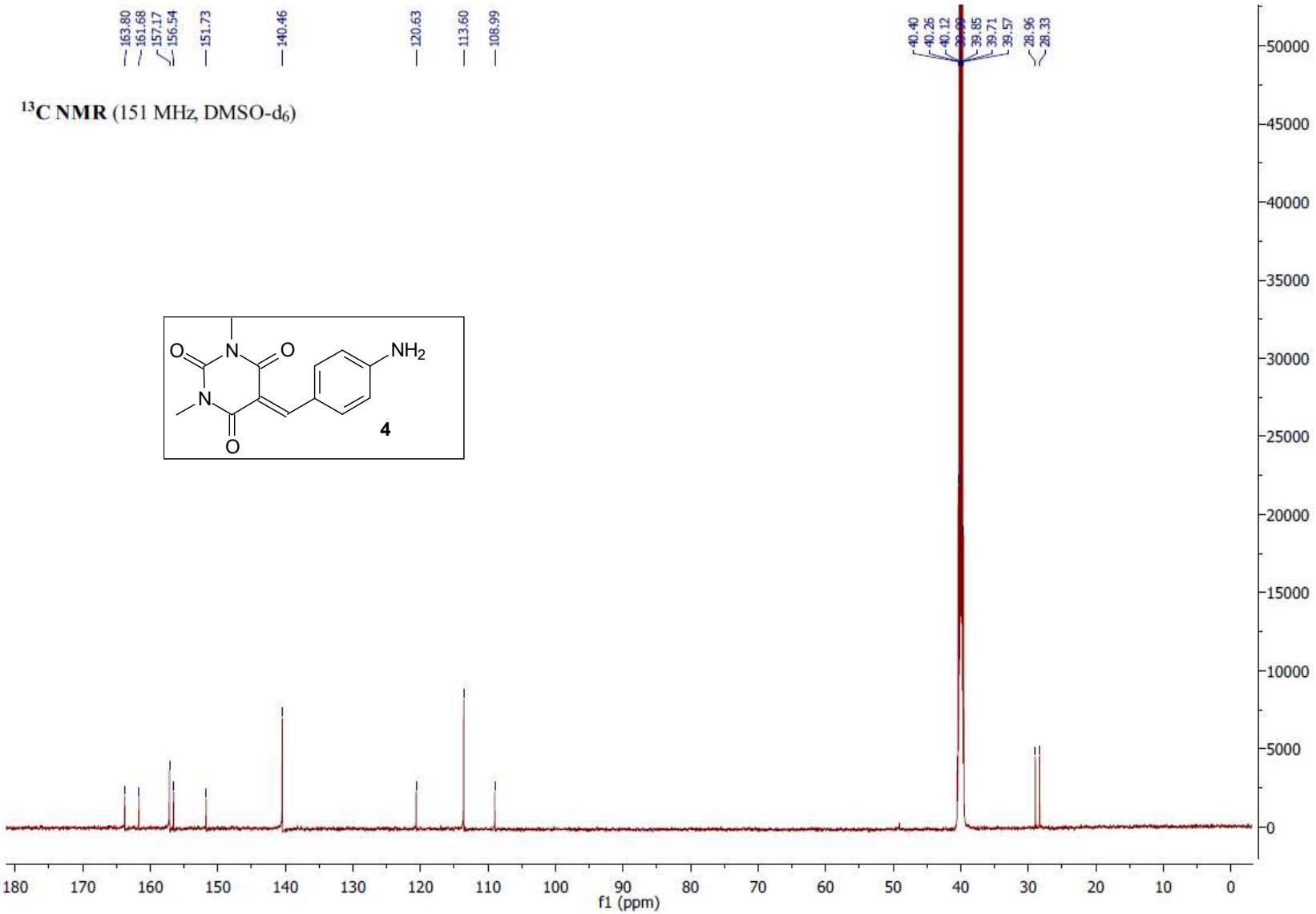
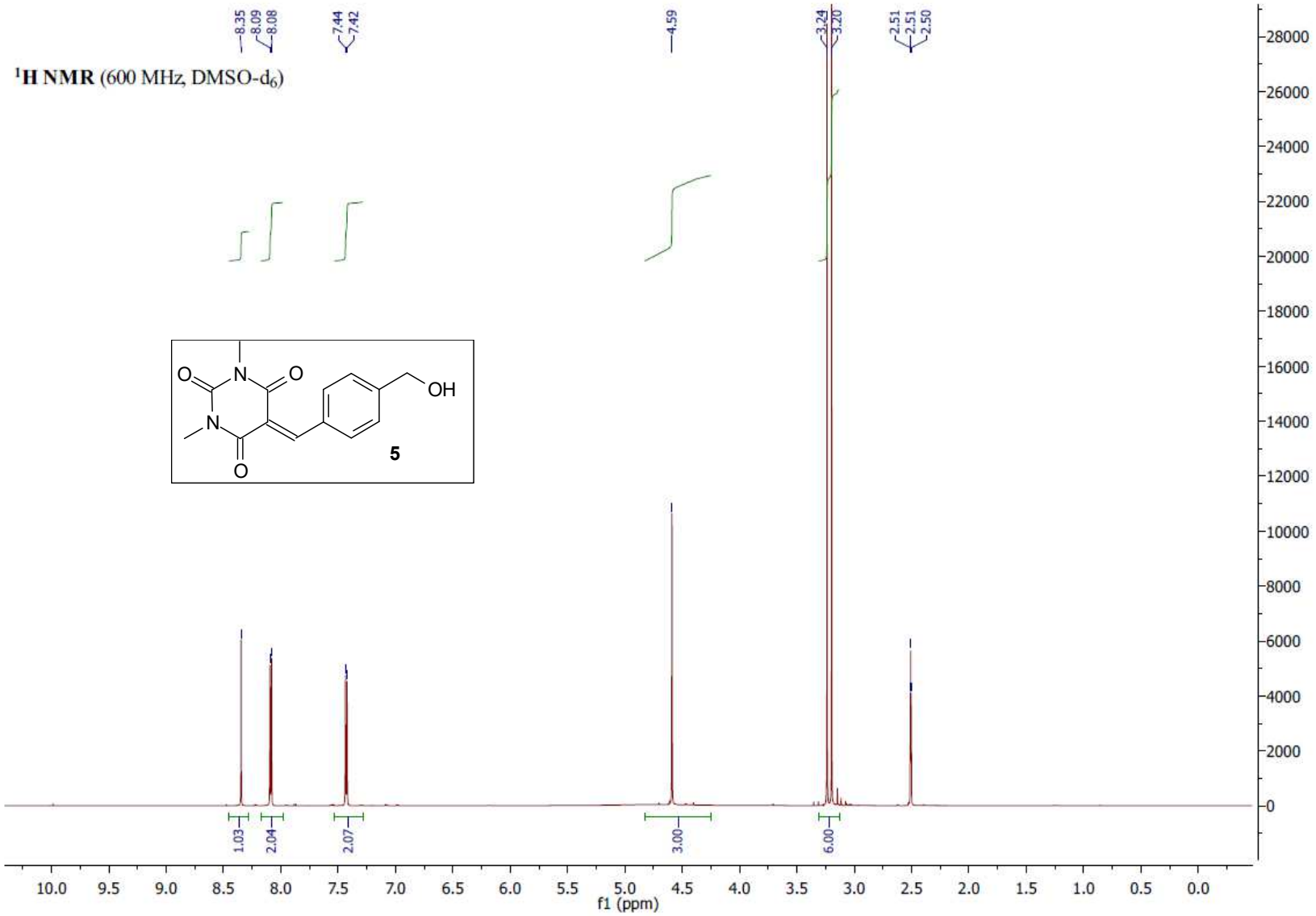
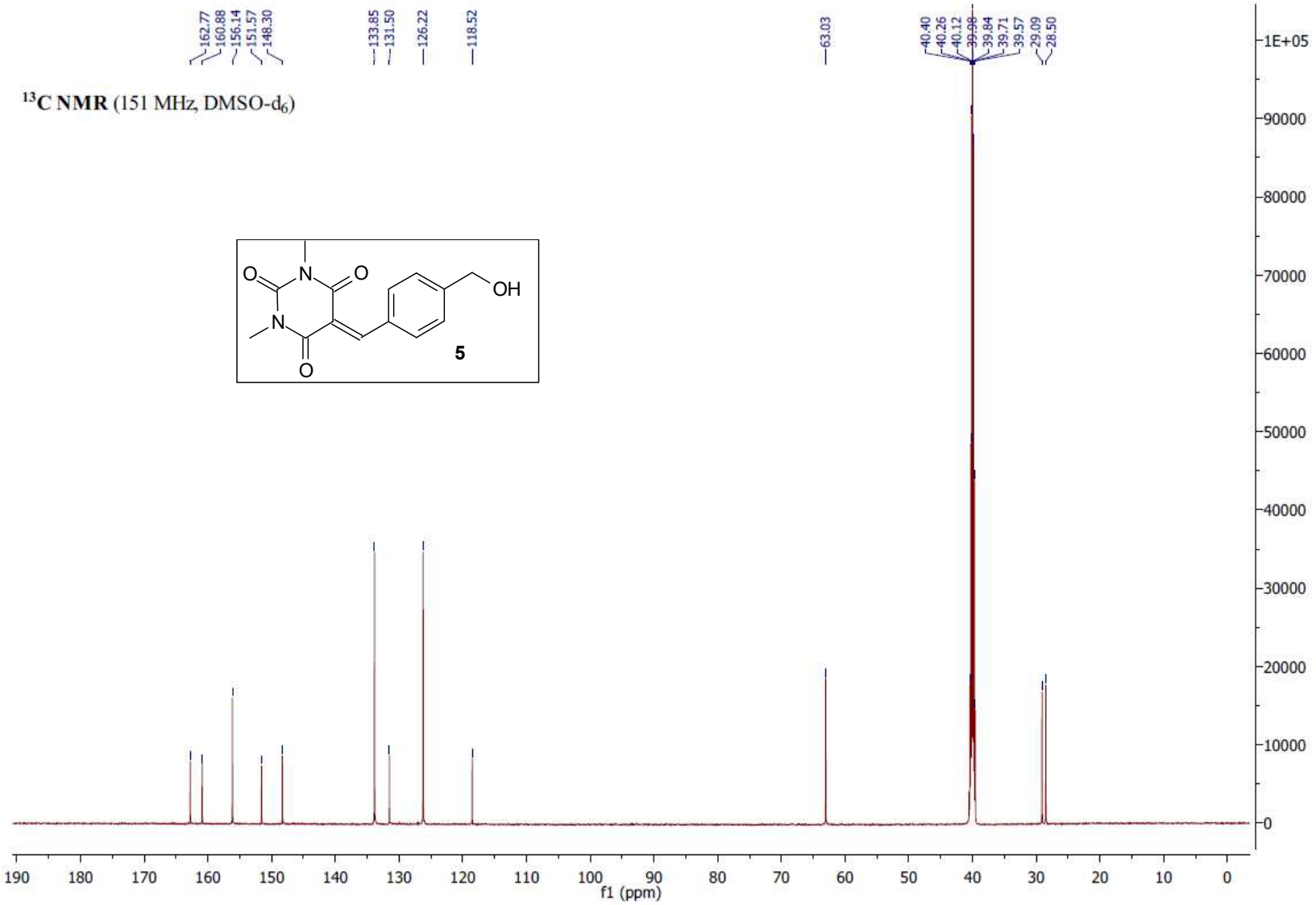


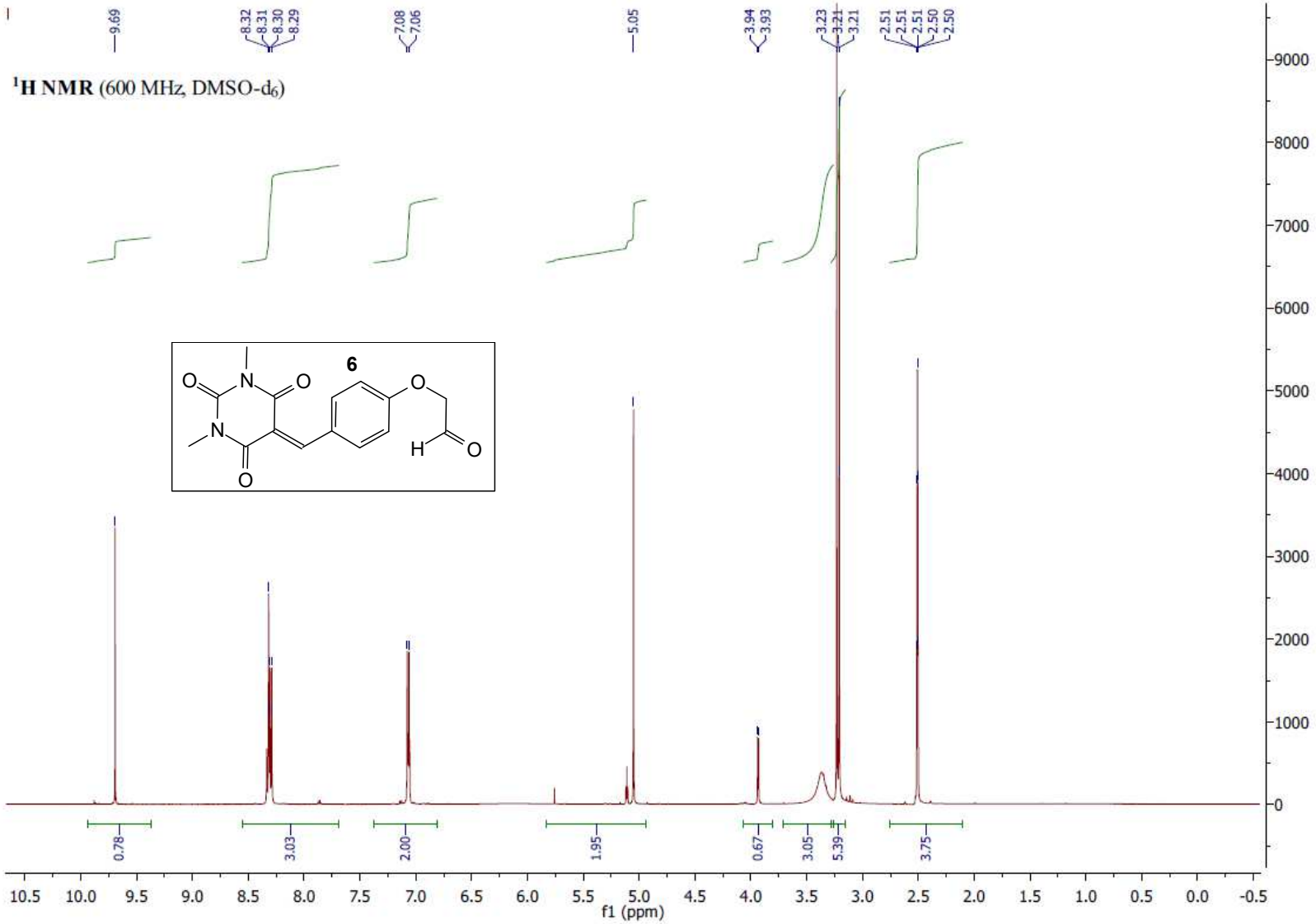
Fig. S20 Fluorescence labeling studies. U-2 OS cells were first treated with either the dienophile-taxol **38** (20 μM) or DMSO for 2 hours, washed, treated with either FITC (33.3 μM) or its heterodiene derivative **30** (33.3 μM) for additional 10 hours, washed again, stained with Hoechst 33342, and visualized with fluorescence microscopy.

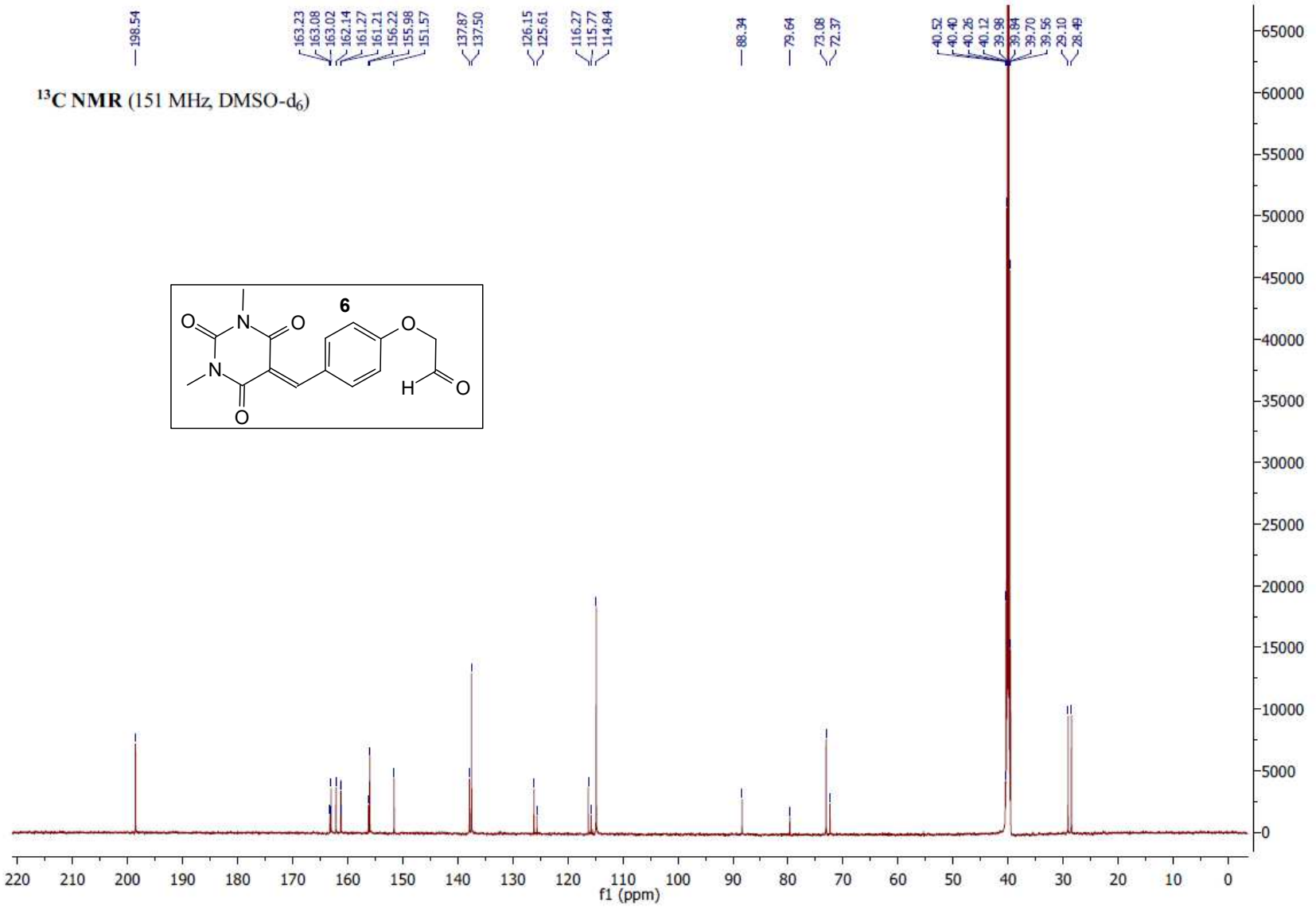

8.34
8.32
8.18
7.03
6.65
6.64
3.34
3.22
2.51
2.51
2.51
2.50
2.50
1H NMR (600 MHz, DMSO-d6)
O
N
O
NH2
N
O
4
1.86
1.00
2.01
2.01
8.52
5.80
10.25
f1 (ppm)

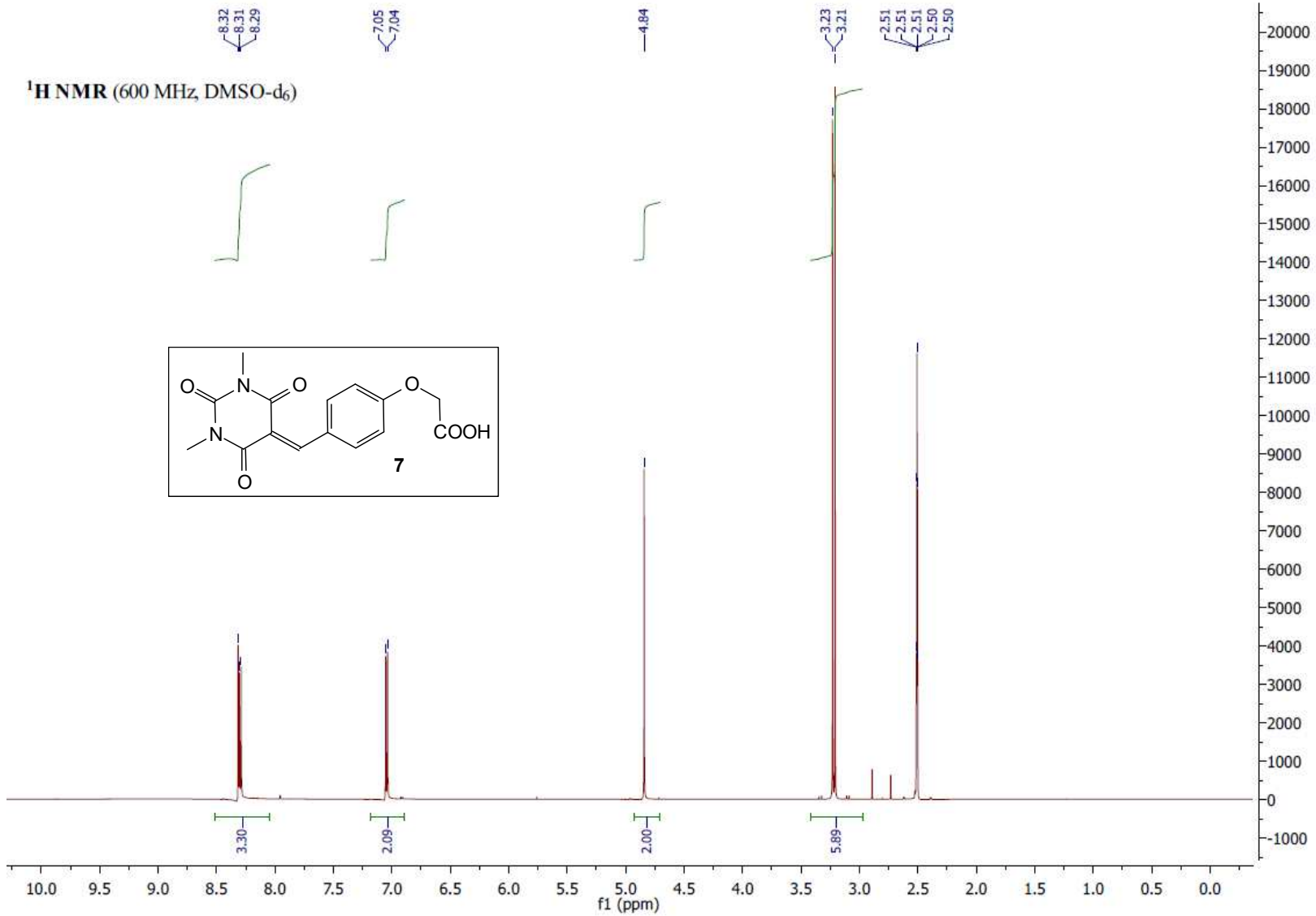

163.80
161.68
157.17
156.54
151.73
140.46
120.63
113.60
108.99
40.40
40.26
40.12
39.98
39.85
39.71
39.57
28.96
28.33
13C NMR (151 MHz, DMSO-d6)
O
N
O
NH2
N
O
4
50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)


8.35
8.09
8.08
7.44
7.42
4.59
3.24
3.20
2.51
2.51
2.50
1H NMR (600 MHz, DMSO-d6)
O
N
O
N
O
OH
5
1.03
2.04
2.07
3.00
6.00
f1 (ppm)

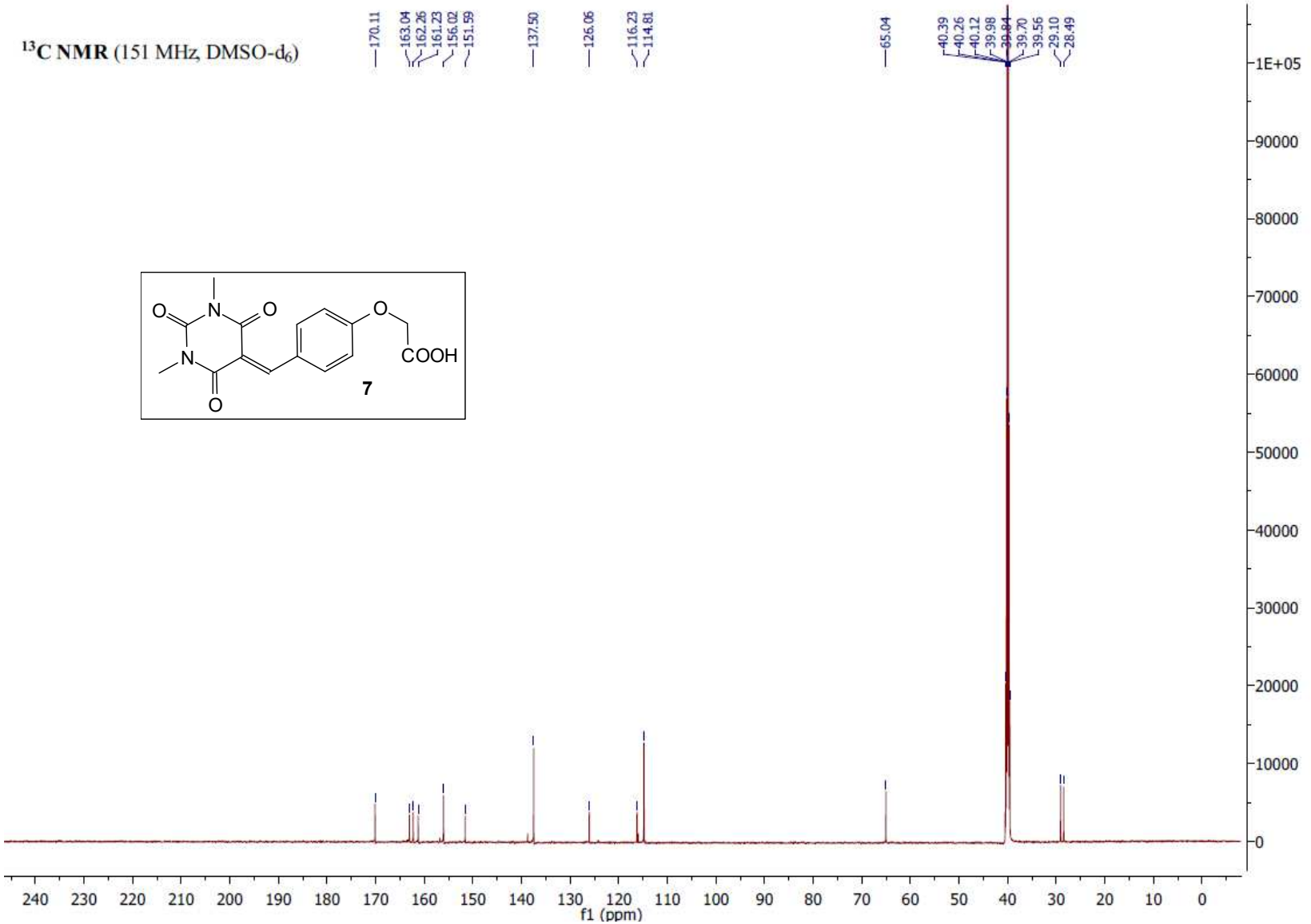

162.77
160.88
156.14
151.57
148.30
133.85
131.50
126.22
118.52
63.03
40.40
40.26
40.12
39.98
39.84
39.71
39.57
29.09
28.50
^{13}C NMR (151 MHz, DMSO-d$_6$)
O
N
O
N
O
OH
5
1E+05
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)


9.69
8.32
8.31
8.30
8.29
7.08
7.06
5.05
3.94
3.93
3.23
3.22
3.21
2.51
2.51
2.51
2.50
2.50
¹H NMR (600 MHz, DMSO-d6)
6
0.78
3.03
2.00
1.95
0.67
3.05
5.39
3.75
f1 (ppm)


198.54
163.23
163.08
163.02
162.14
161.27
161.21
156.22
155.98
151.57
137.87
137.50
126.15
125.61
116.27
115.77
114.84
88.34
79.64
73.08
72.37
40.52
40.40
40.26
40.12
39.98
39.84
39.70
39.56
29.10
28.49
13C NMR (151 MHz, DMSO-d6)
6
f1 (ppm)

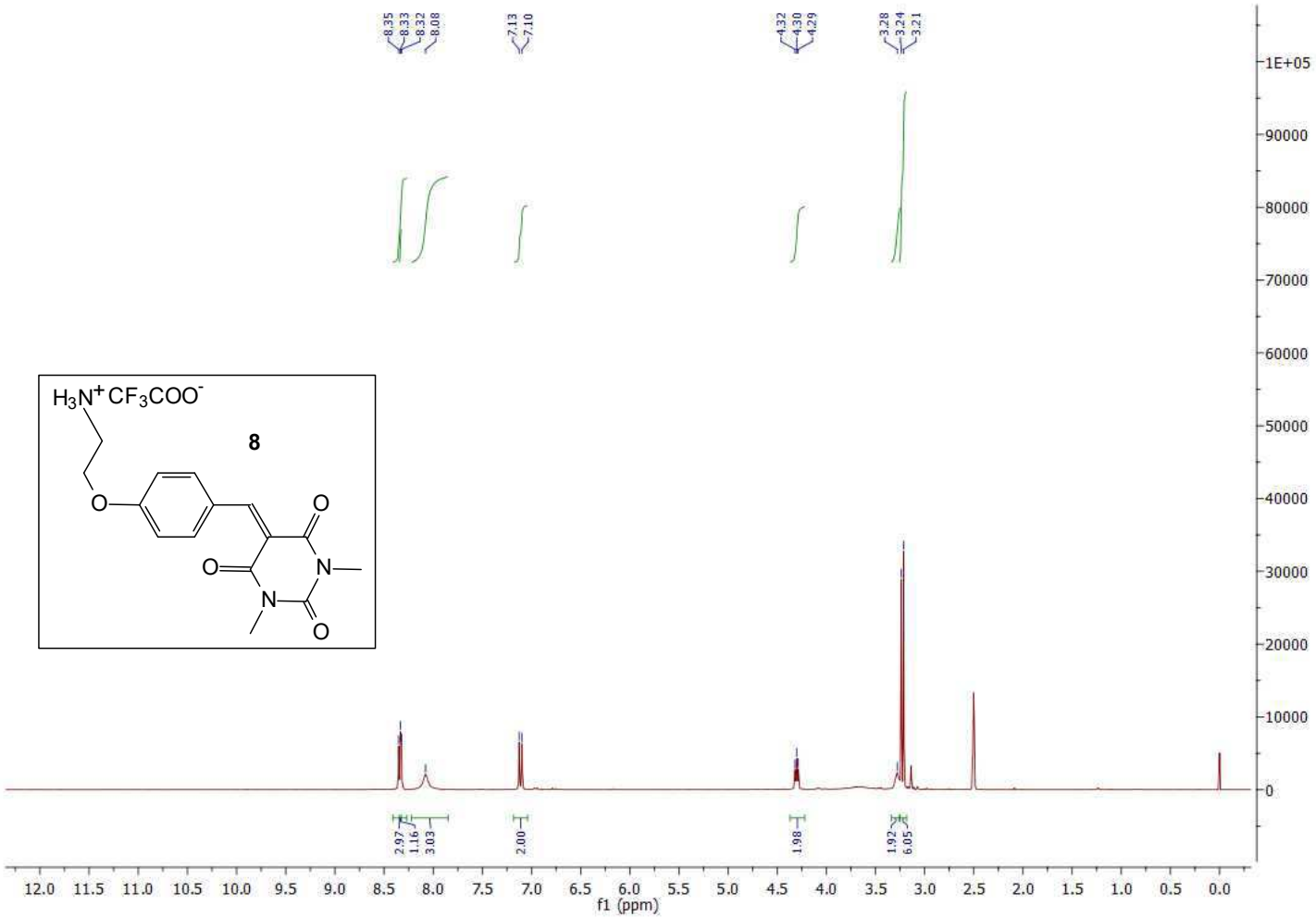

8.32
8.31
8.29
7.05
7.04
4.84
3.23
3.21
2.51
2.51
2.51
2.50
2.50
^{1}H NMR (600 MHz, DMSO-d$_6$)
O
N
O
N
O
O
COOH
7
3.30
2.09
2.00
5.89
f1 (ppm)

^{13}C NMR (151 MHz, DMSO-d_6)

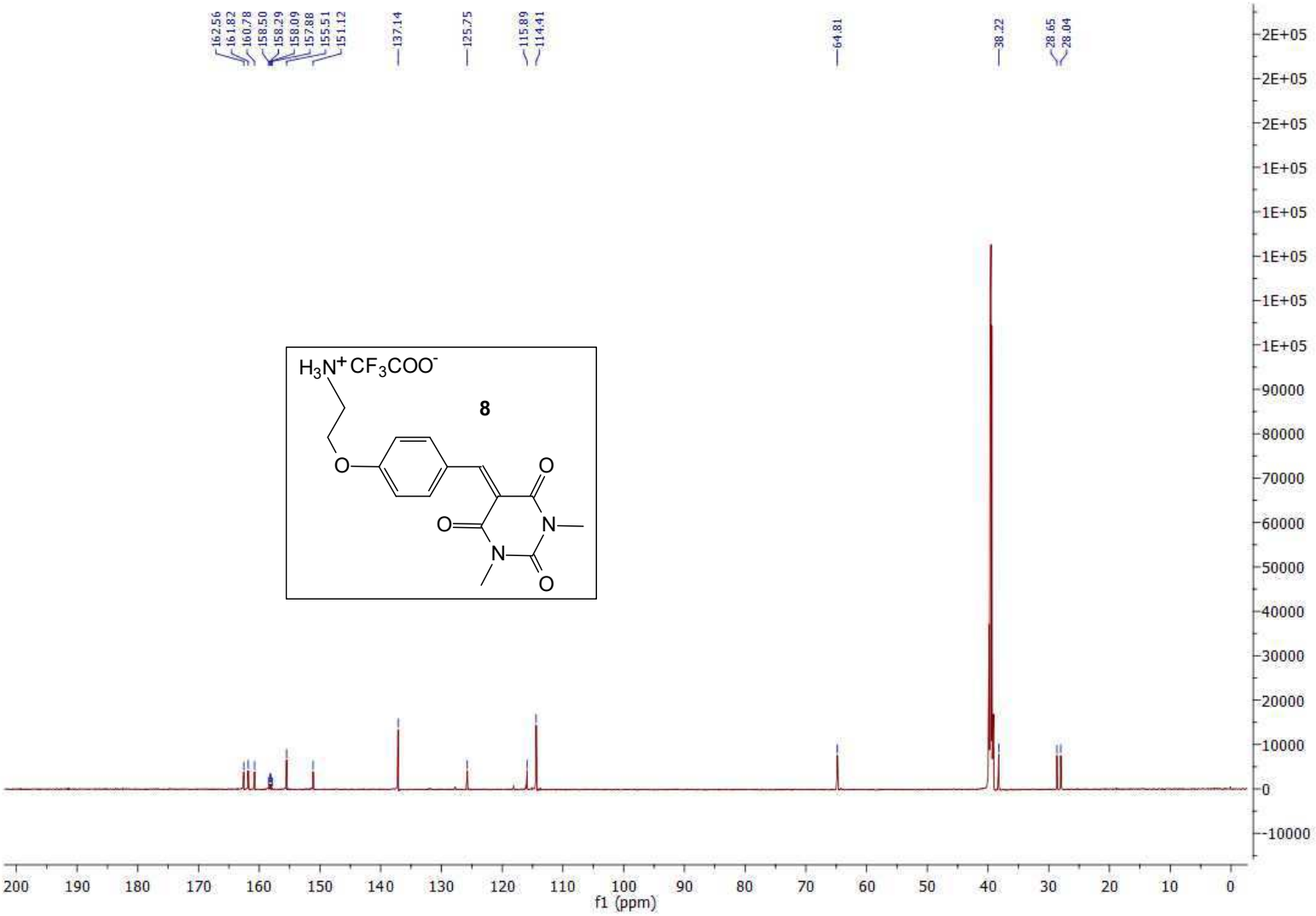


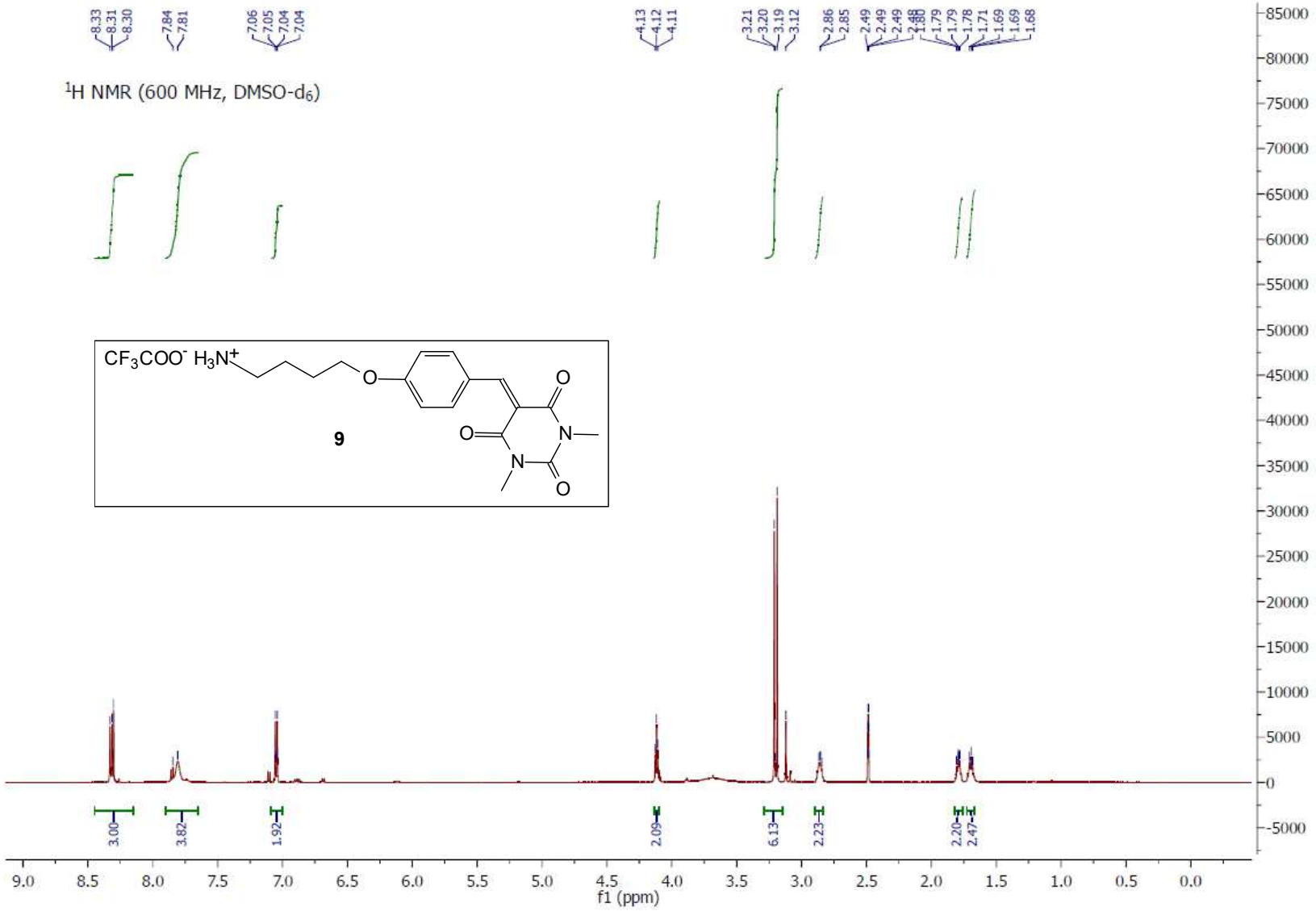
7

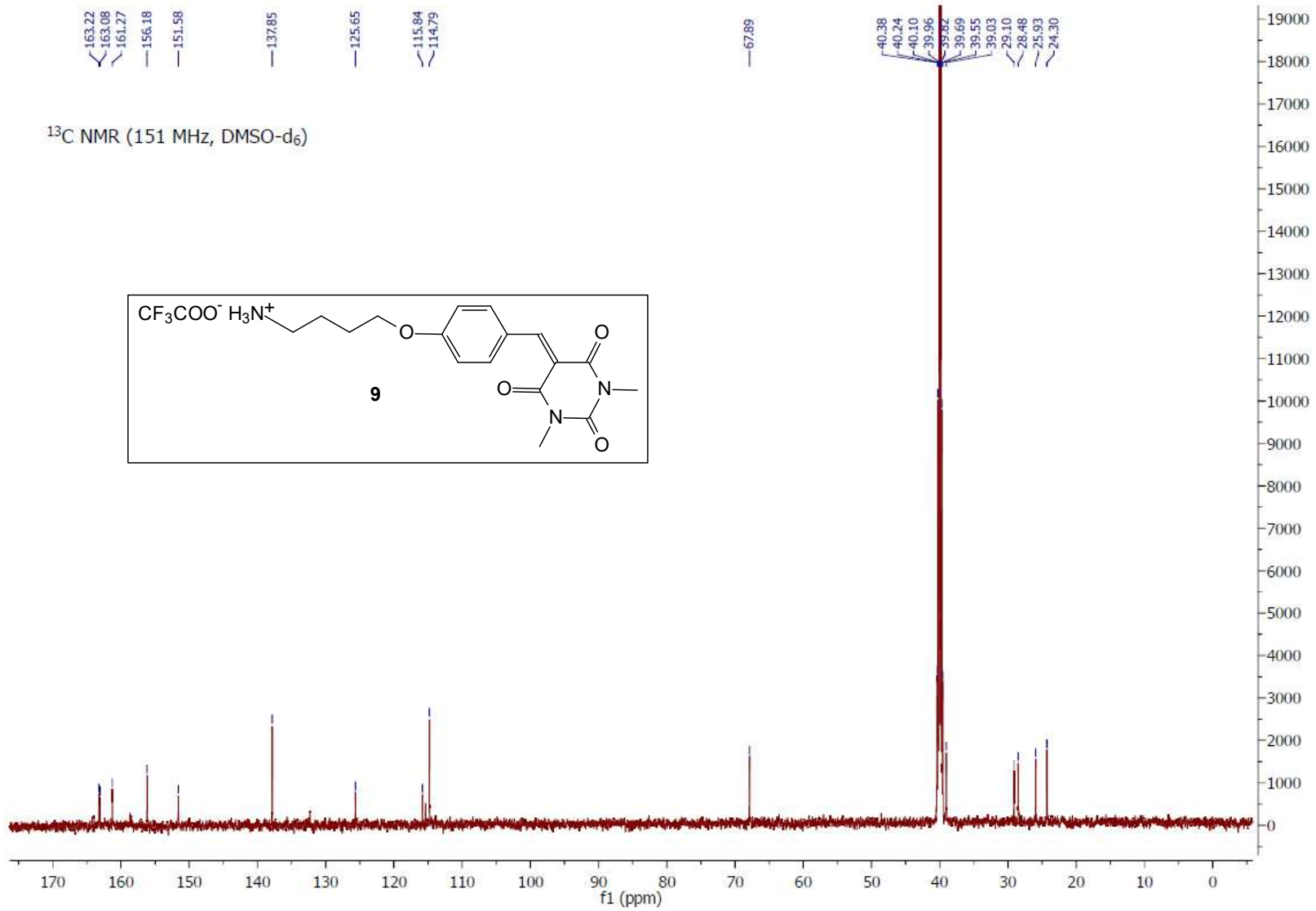
^{1}H NMR (300 MHz, DMSO-d_6)

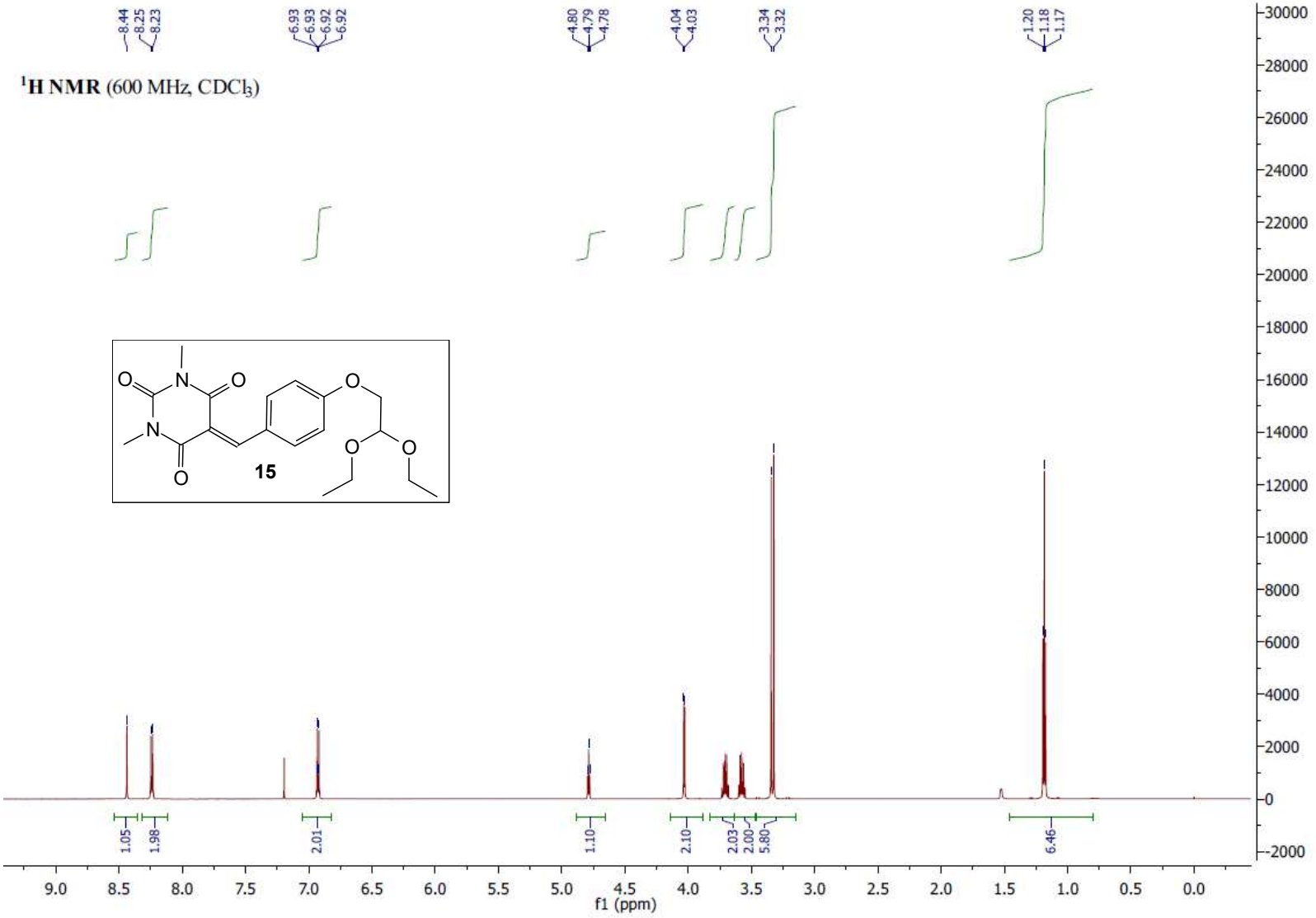


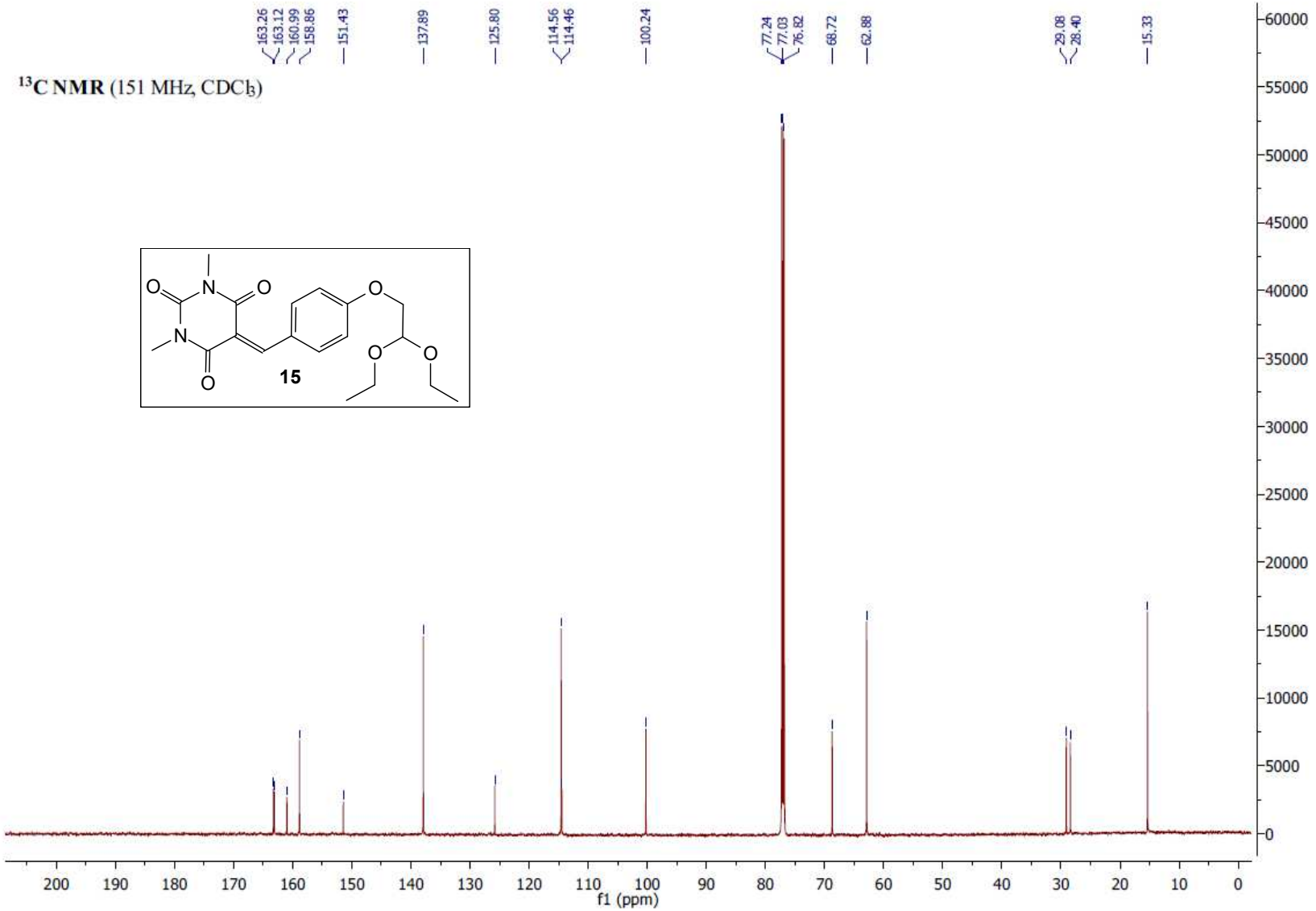
^{13}C NMR (151 MHz, DMSO-d_6)



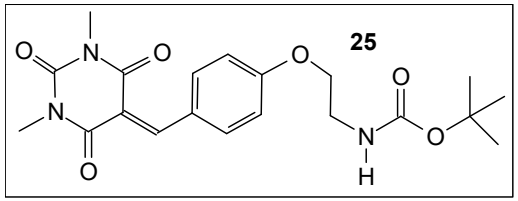

8.33
8.31
8.30
7.84
7.81
7.06
7.05
7.04
7.04
4.13
4.12
4.11
3.21
3.20
3.19
3.12
2.86
2.85
2.49
2.49
2.49
2.48
1.80
1.79
1.79
1.78
1.71
1.69
1.69
1.68
^{1}H NMR (600 MHz, DMSO-d$_6$)
CF_3COO^- H_3N^+
O
O
N
O
N
O
9
3.00
3.82
1.92
2.09
6.13
2.23
2.20
2.47
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1 (ppm)
85000
80000
75000
70000
65000
60000
55000
50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
-5000


163.22
163.08
161.27
156.18
151.58
137.85
125.65
115.84
114.79
67.89
40.38
40.24
40.10
39.96
39.82
39.69
39.55
39.03
29.10
28.48
25.93
24.30
^{13}C NMR (151 MHz, DMSO-d$_6$)
CF_3COO^- H_3N^+
O
O
N
N
O
O
9
19000
18000
17000
16000
15000
14000
13000
12000
11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

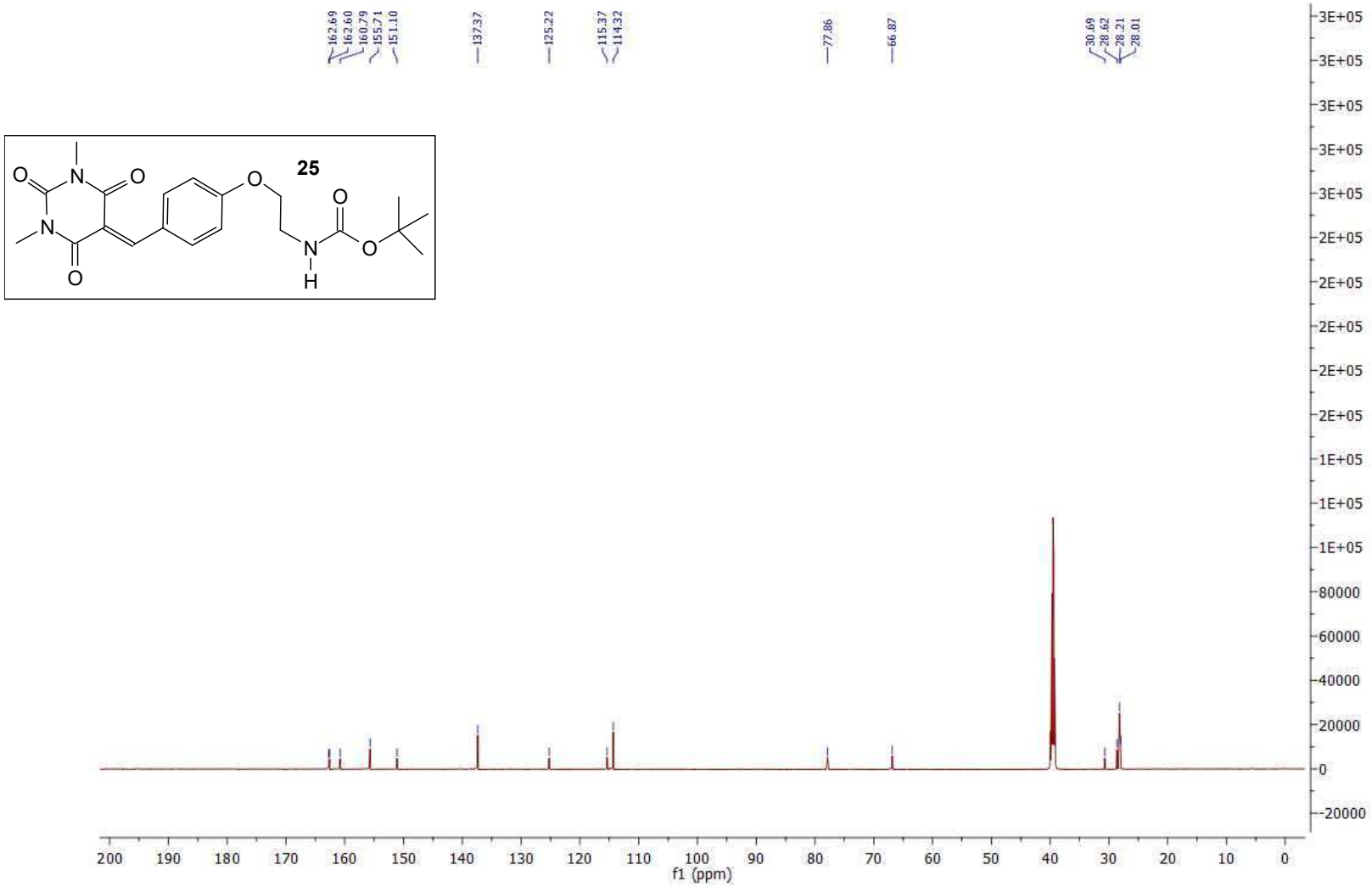

8.44
8.25
8.23
6.93
6.93
6.92
6.92
4.80
4.79
4.78
4.04
4.03
3.34
3.32
1.20
1.18
1.17
1H NMR (600 MHz, CDCl3)
15
1.05
1.98
2.01
1.10
2.10
2.03
2.00
5.80
6.46
f1 (ppm)

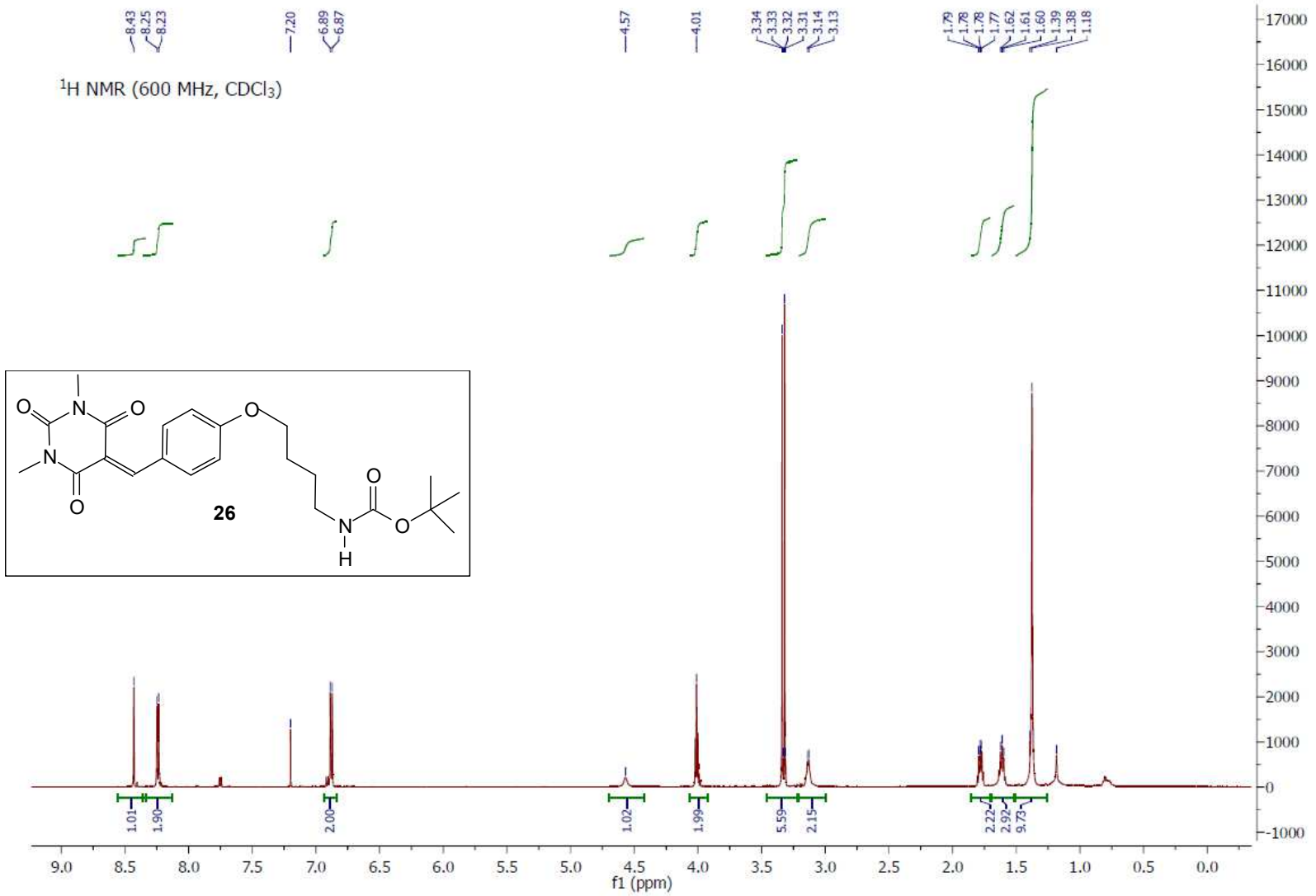

163.26
163.12
160.99
158.86
151.43
137.89
125.80
114.56
114.46
100.24
77.24
77.03
76.82
68.72
62.88
29.08
28.40
15.33
^{13}C NMR (151 MHz, CDCl$_3$)
15
60000
55000
50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)

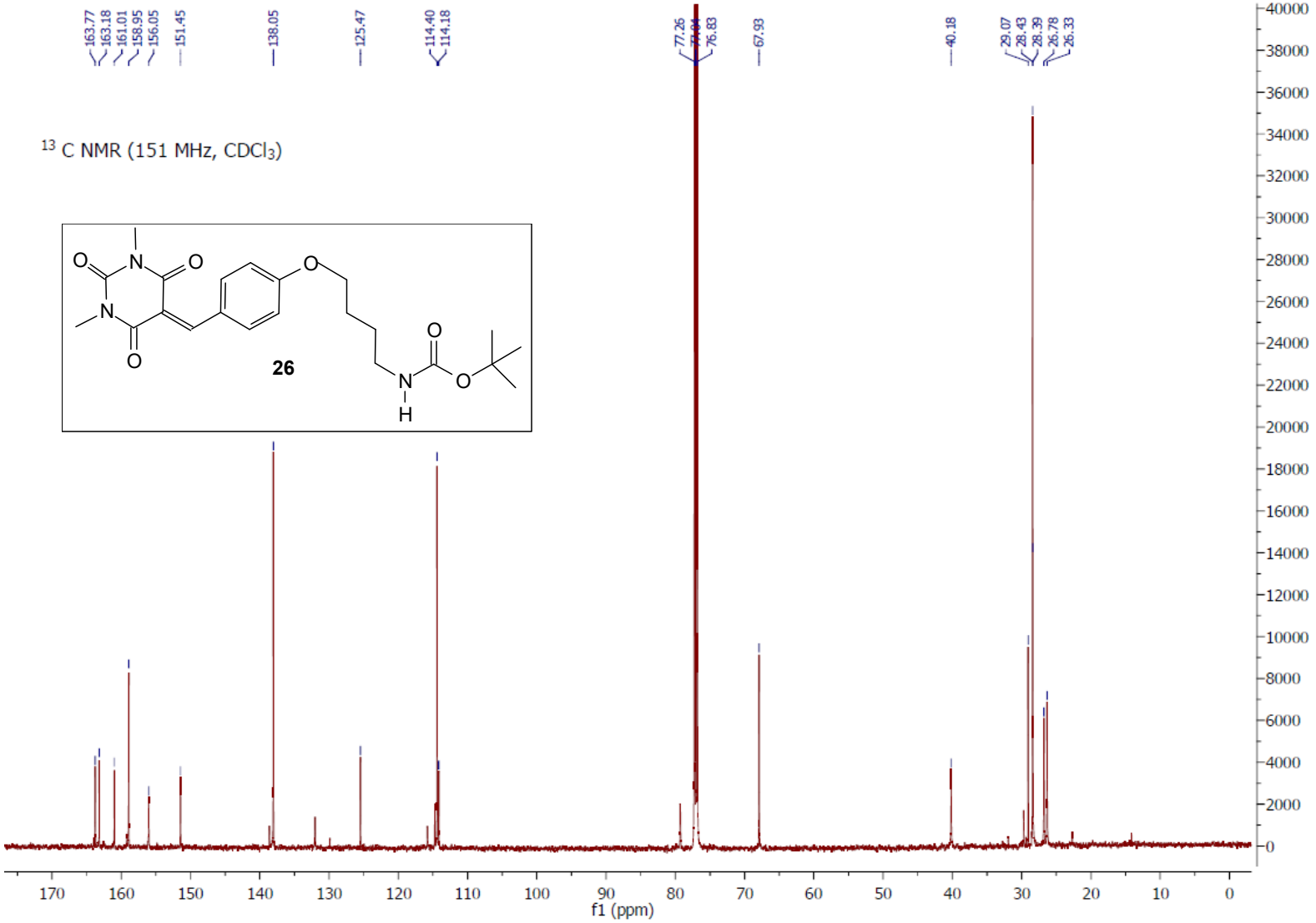
^{1}H NMR (300 MHz, DMSO-d_6)

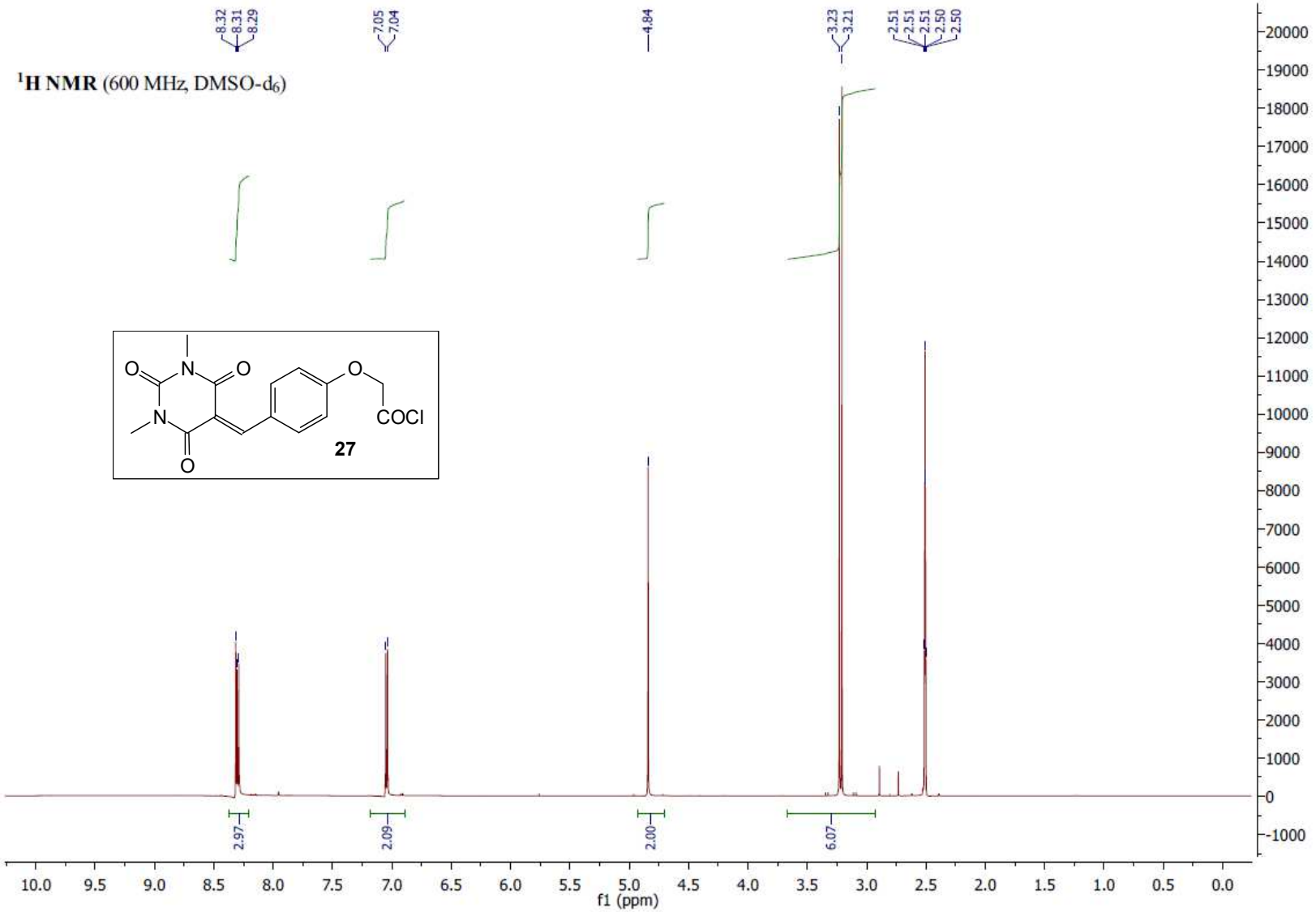


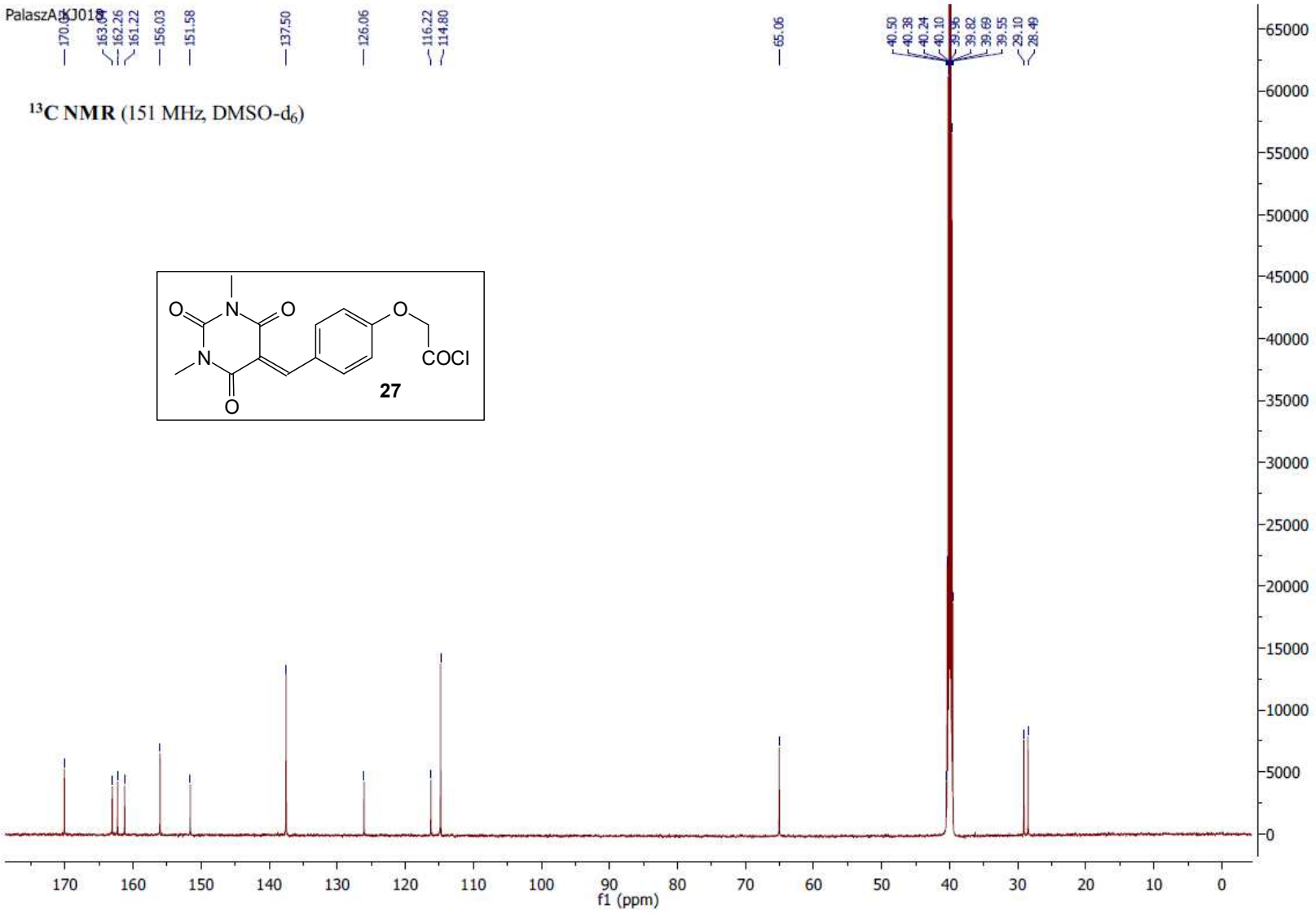
^{13}C NMR (151 MHz, DMSO-d_6)




^{1}H NMR (600 MHz, $CDCl_3$)
8.43
8.25
8.23
7.20
6.89
6.87
4.57
4.01
3.34
3.33
3.32
3.31
3.14
3.13
1.79
1.76
1.78
1.77
1.62
1.61
1.60
1.39
1.38
1.18
26
1.01
1.90
2.00
1.02
1.99
5.59
2.15
2.22
2.92
9.73
f1 (ppm)

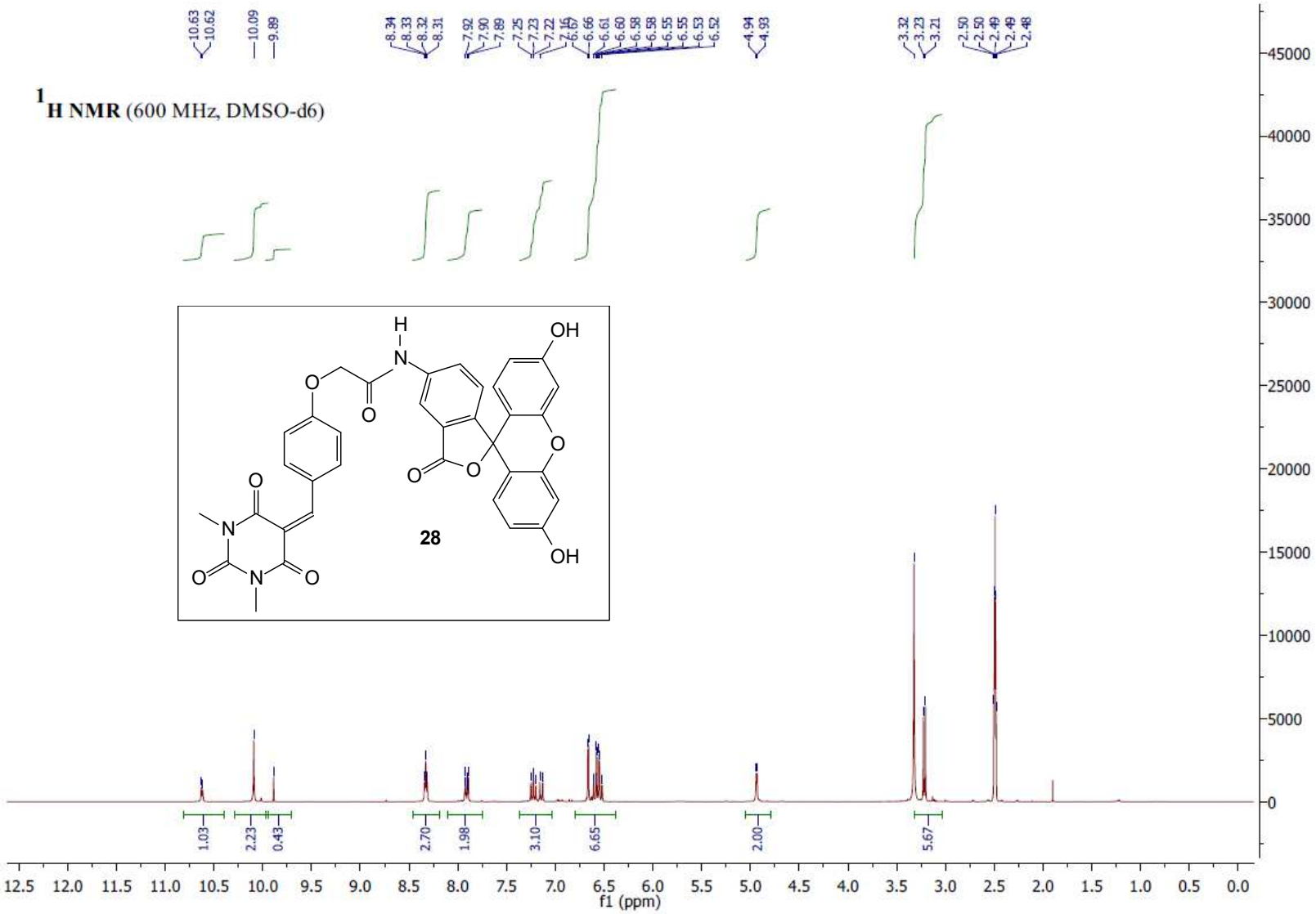

163.77
163.18
161.01
158.95
156.05
151.45
138.05
125.47
114.40
114.18
77.26
76.83
67.93
40.18
29.07
28.43
28.39
26.78
26.33
13 C NMR (151 MHz, CDCl3)
26
f1 (ppm)

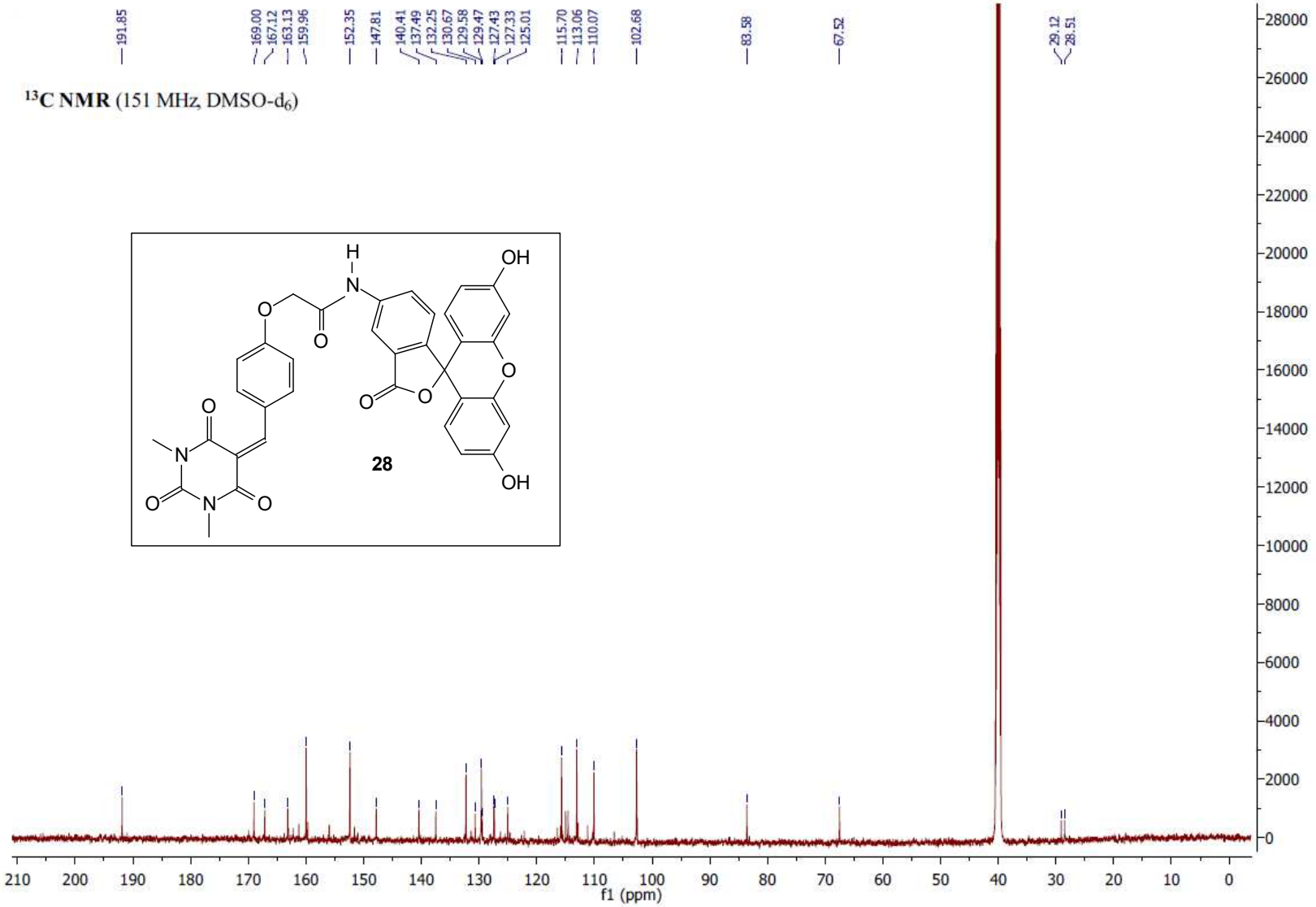

8.32
8.31
8.29
7.05
7.04
4.84
3.23
3.21
2.51
2.51
2.51
2.50
2.50
^{1}H NMR (600 MHz, DMSO-d$_6$)
O
N
O
O
N
O
COCl
27
2.97
2.09
2.00
6.07
f1 (ppm)



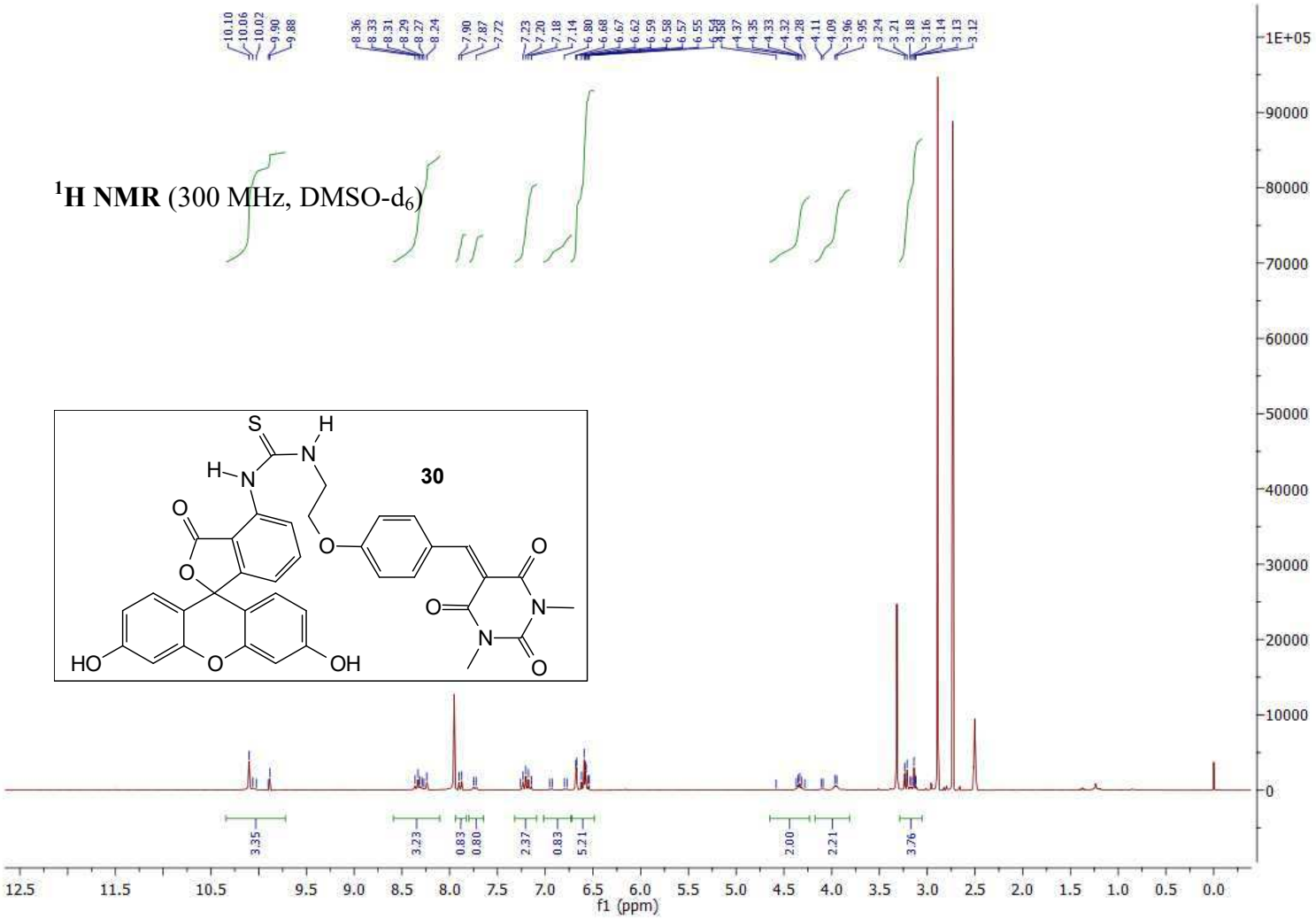
^{13}C NMR (151 MHz, DMSO-d_6)

27

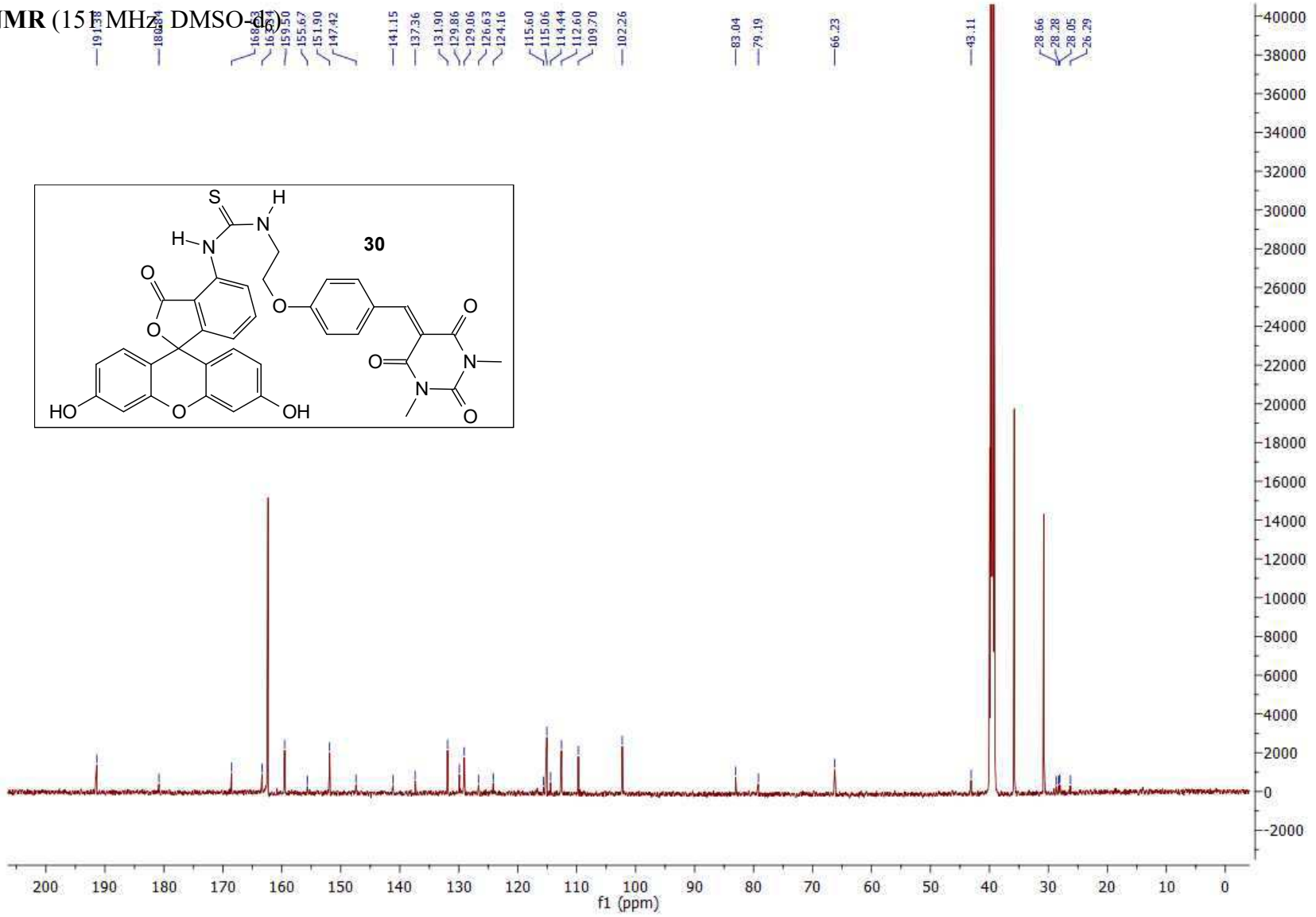

1H NMR (600 MHz, DMSO-d6)
28
f1 (ppm)


191.85
169.00
167.12
163.13
159.96
152.35
147.81
140.41
137.49
132.25
130.67
129.58
129.47
127.43
127.33
125.01
115.70
113.06
110.07
102.68
83.58
67.52
29.12
28.51
13C NMR (151 MHz, DMSO-d6)
28
OH
OH
f1 (ppm)

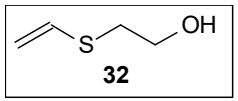
^{1}H NMR (300 MHz, DMSO-d_6)



^{13}C NMR (151 MHz, DMSO-d_6)

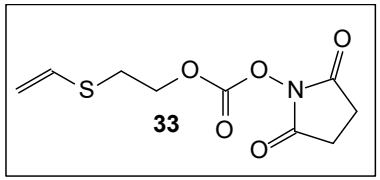


^{1}H NMR (300 MHz, $CDCl_3$

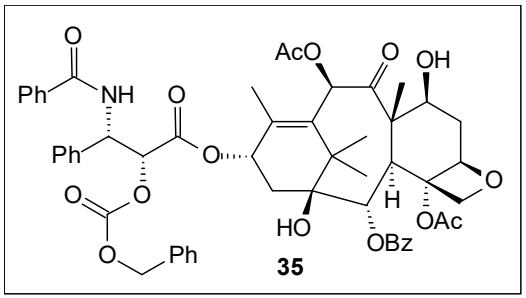


32

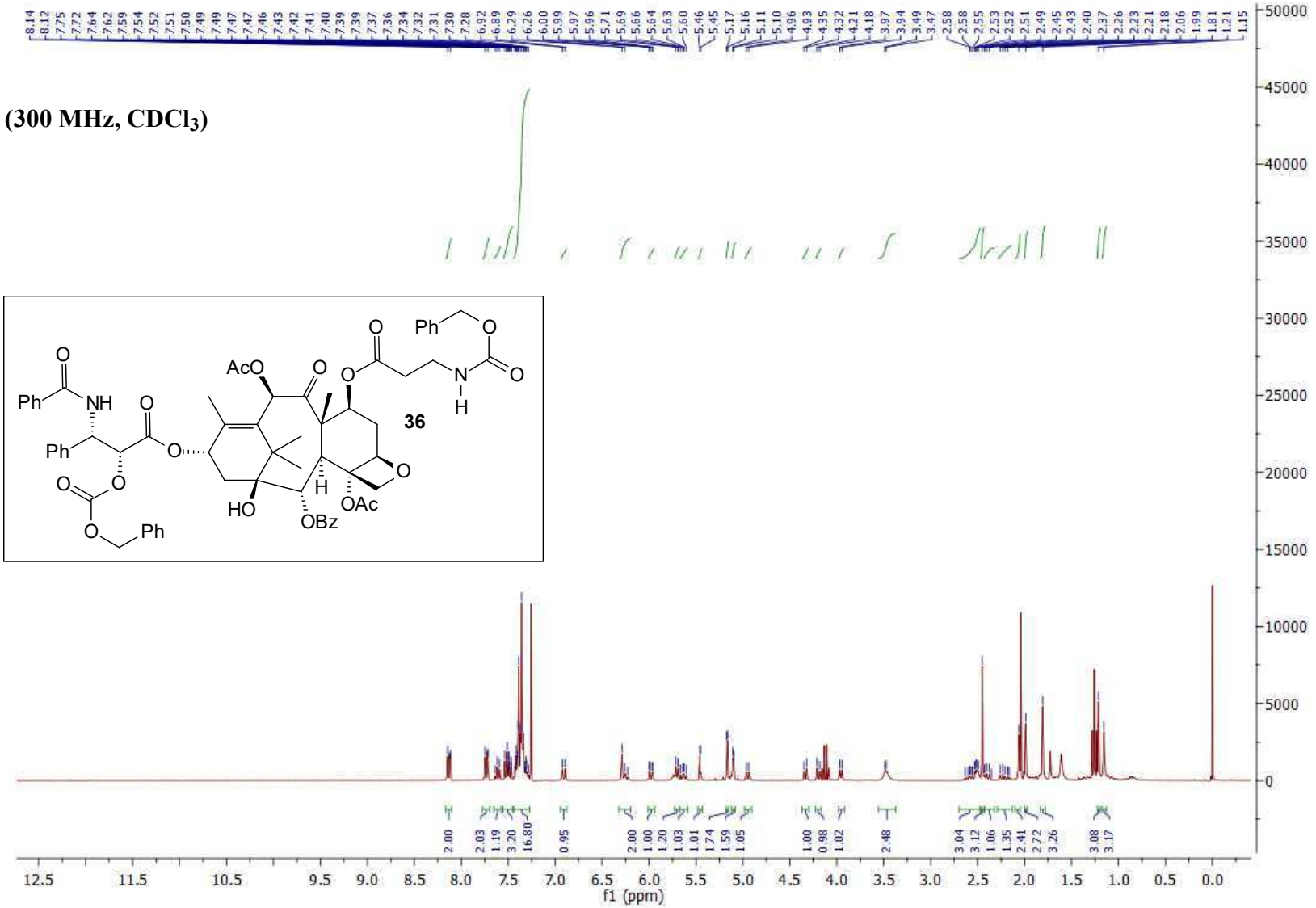
^{1}H NMR (300 MHz, $CDCl_3$



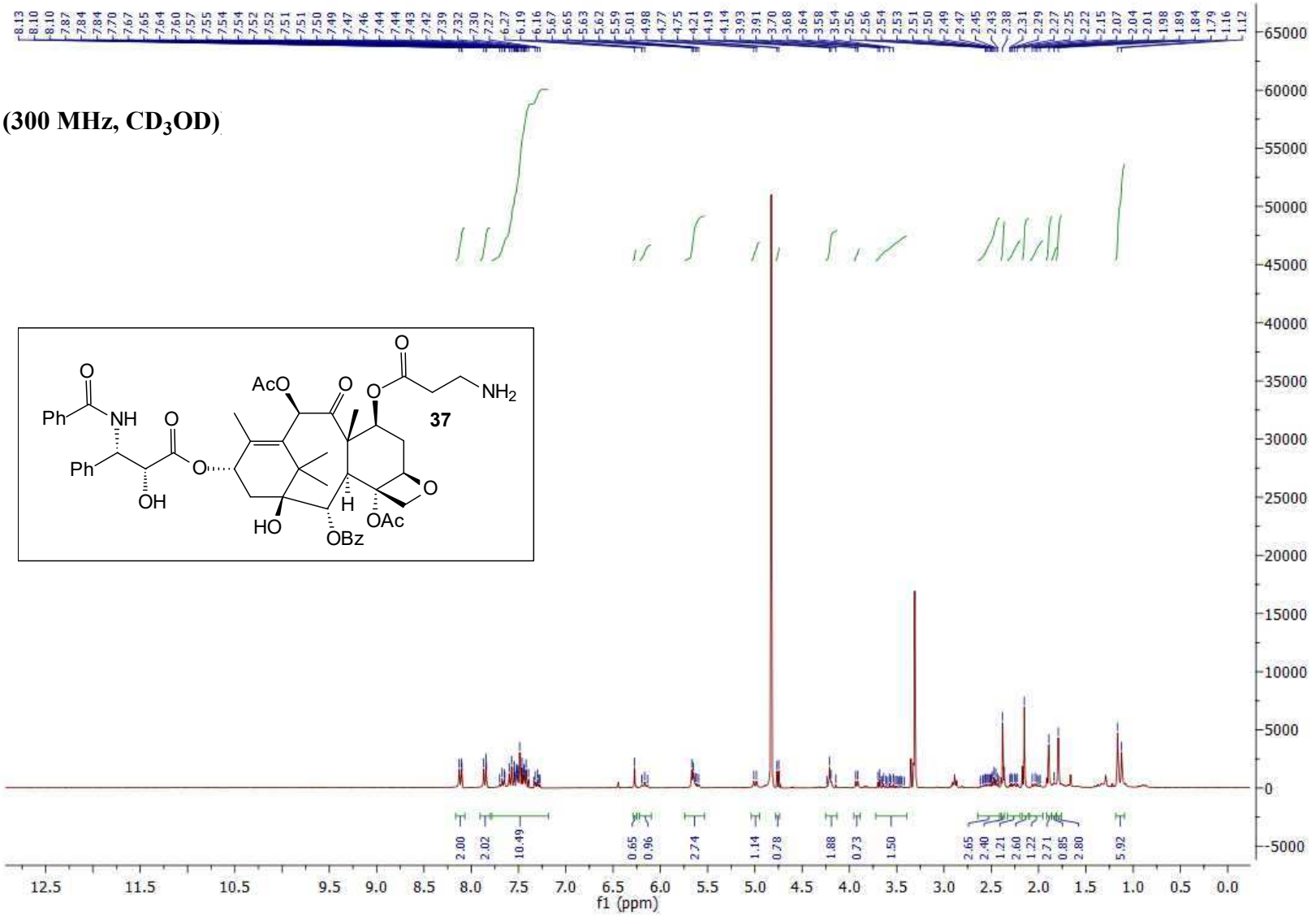
^{1}H NMR (300 MHz, $CDCl_3$)



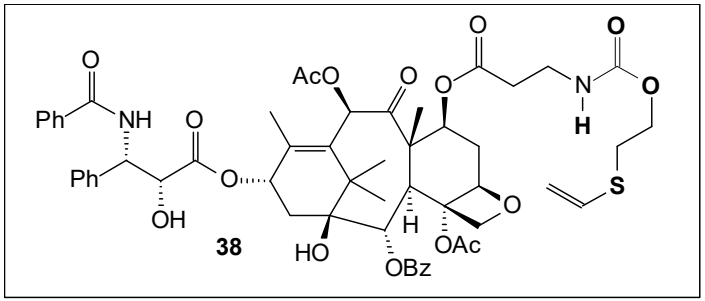
^{1}H NMR (300 MHz, $CDCl_3$)



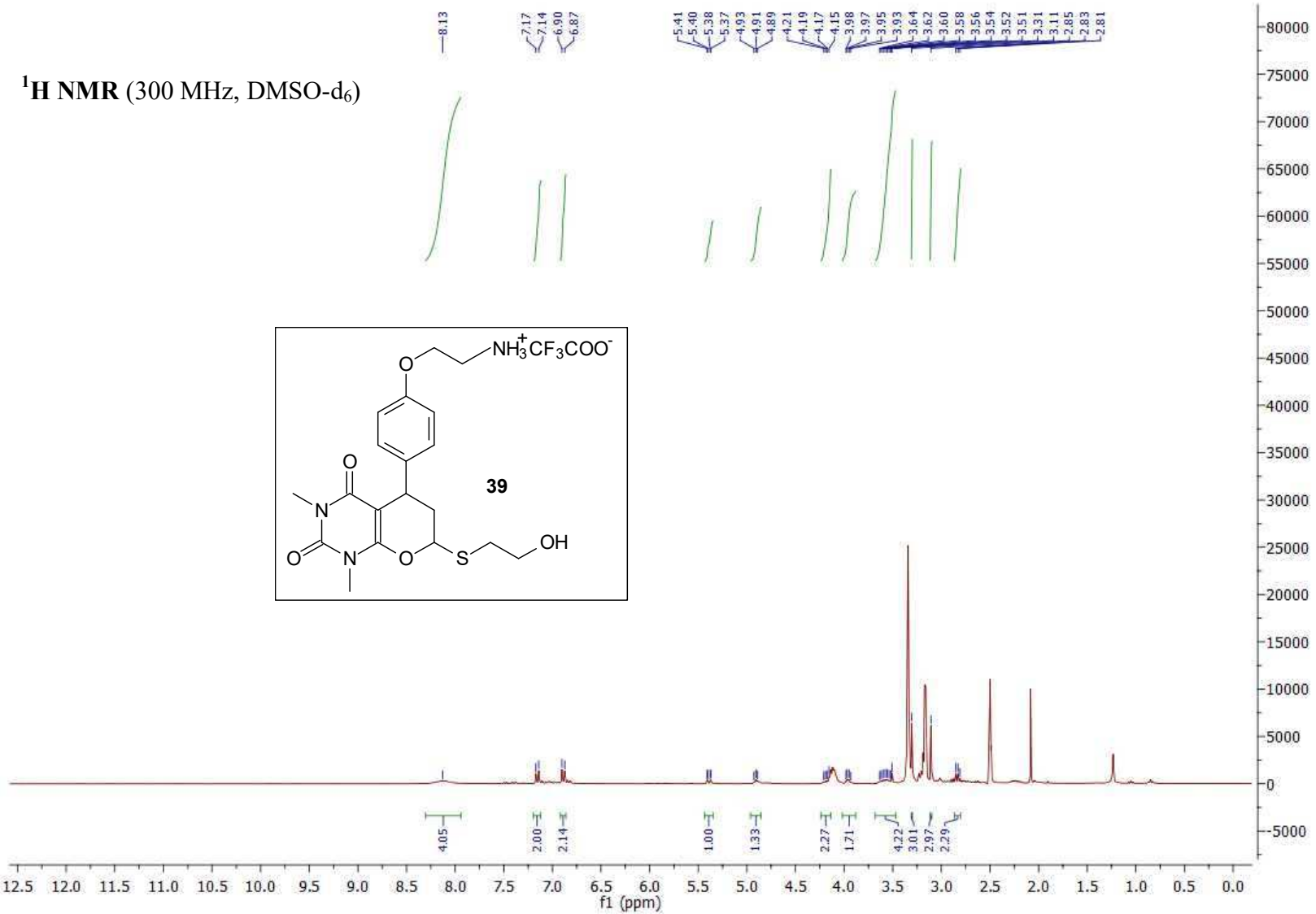
^{1}H NMR (300 MHz, CD_3OD)



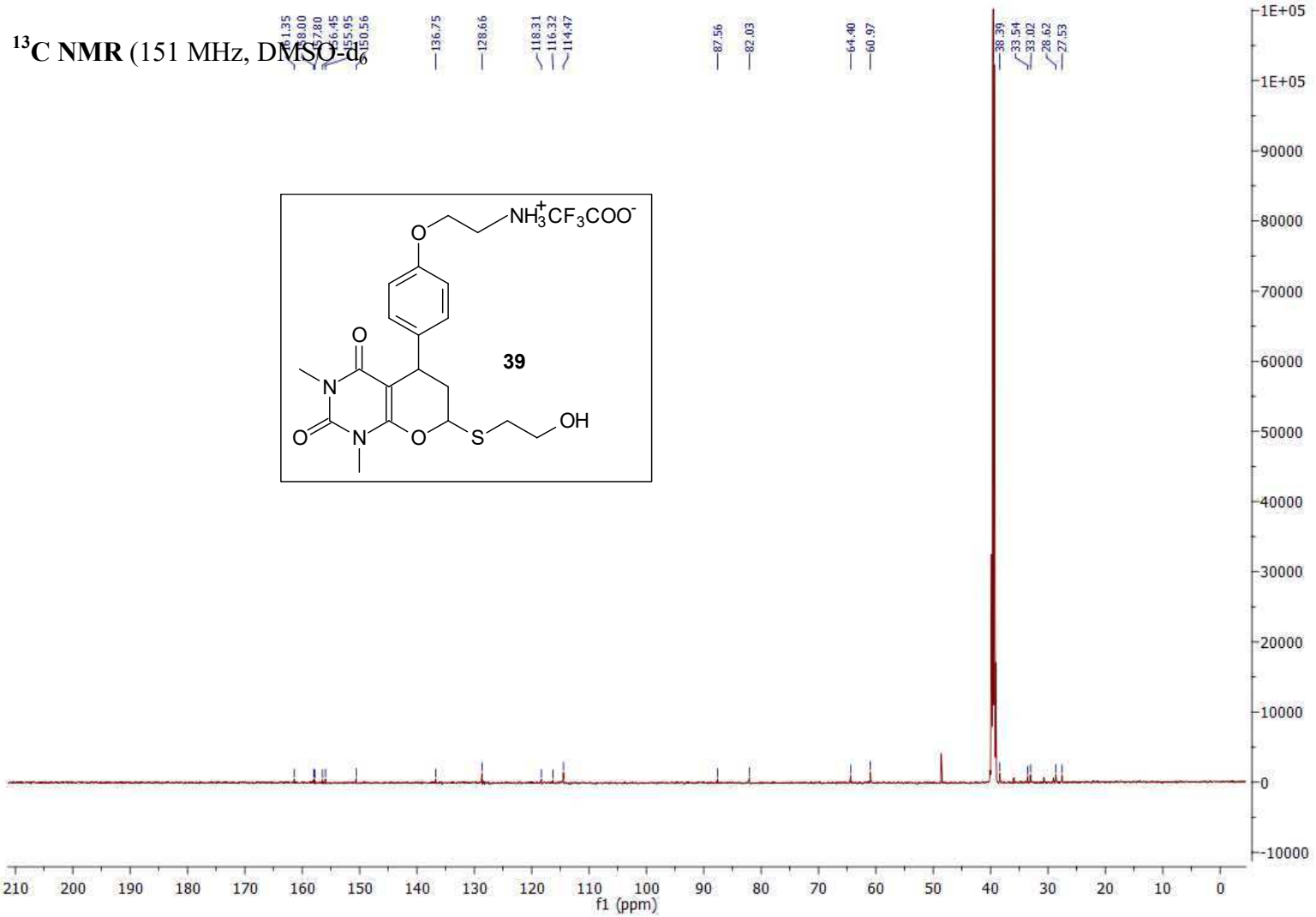
^{1}H NMR (300 MHz, $CDCl_3$)

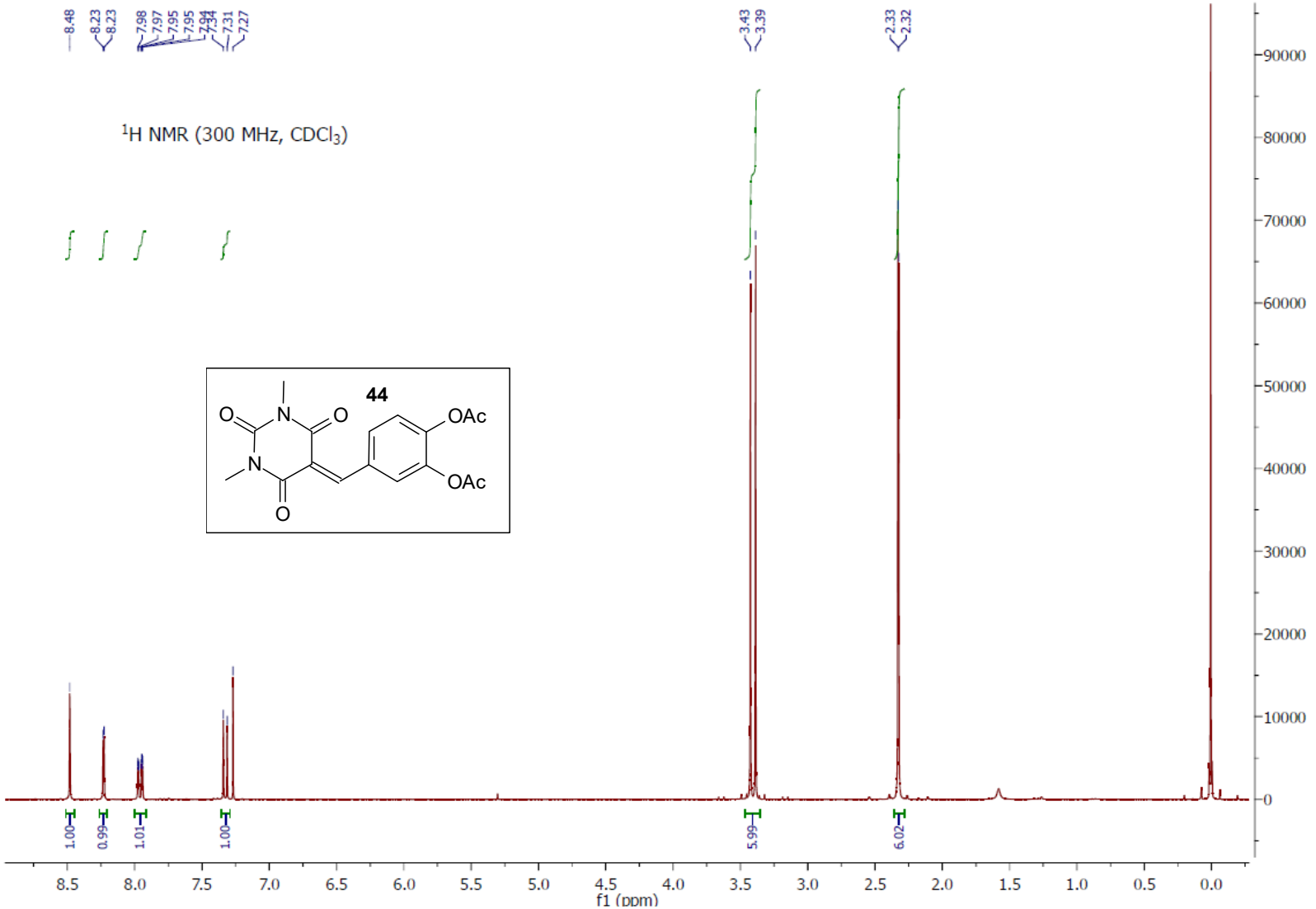


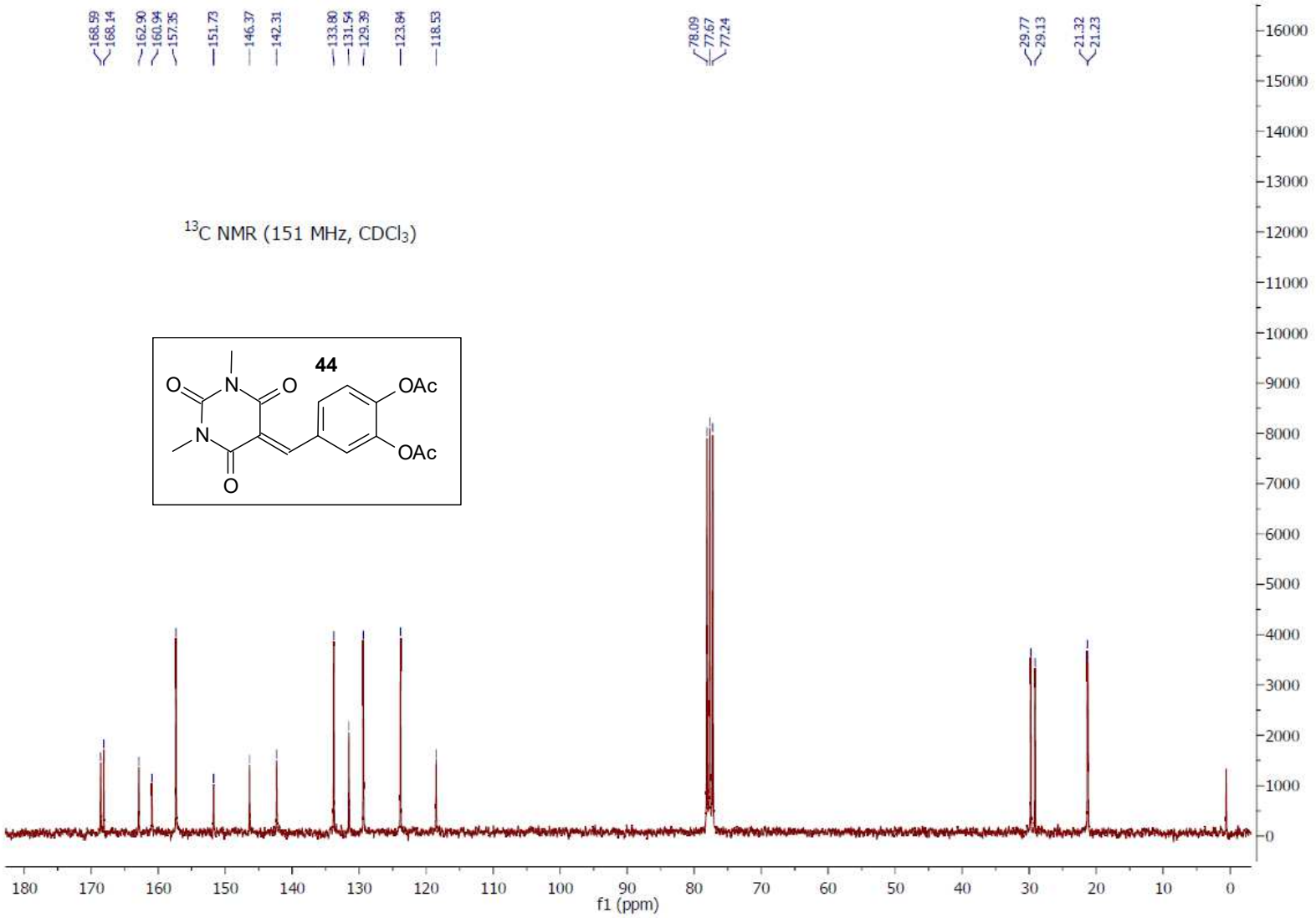
^{1}H NMR (300 MHz, DMSO-d_6)

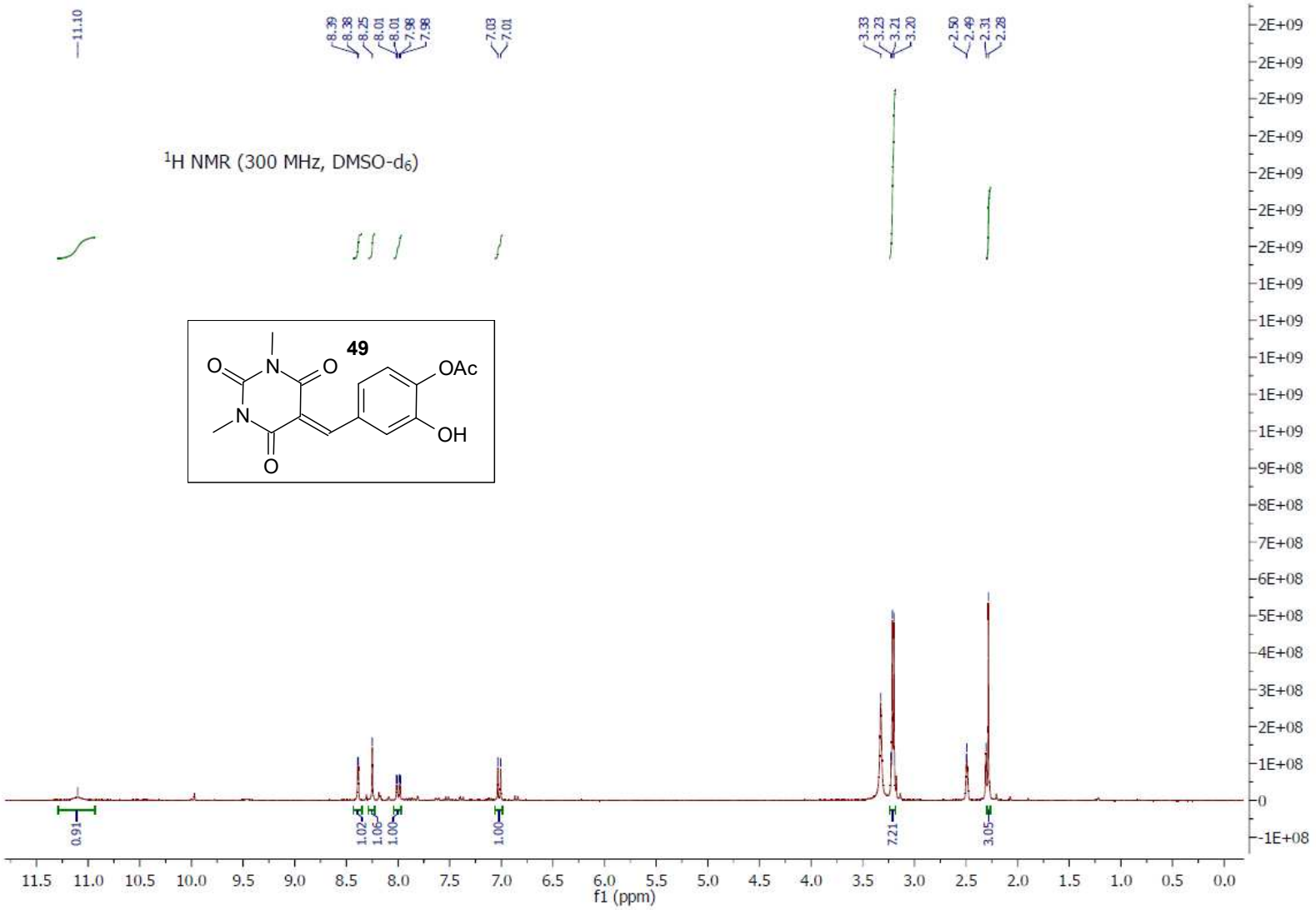


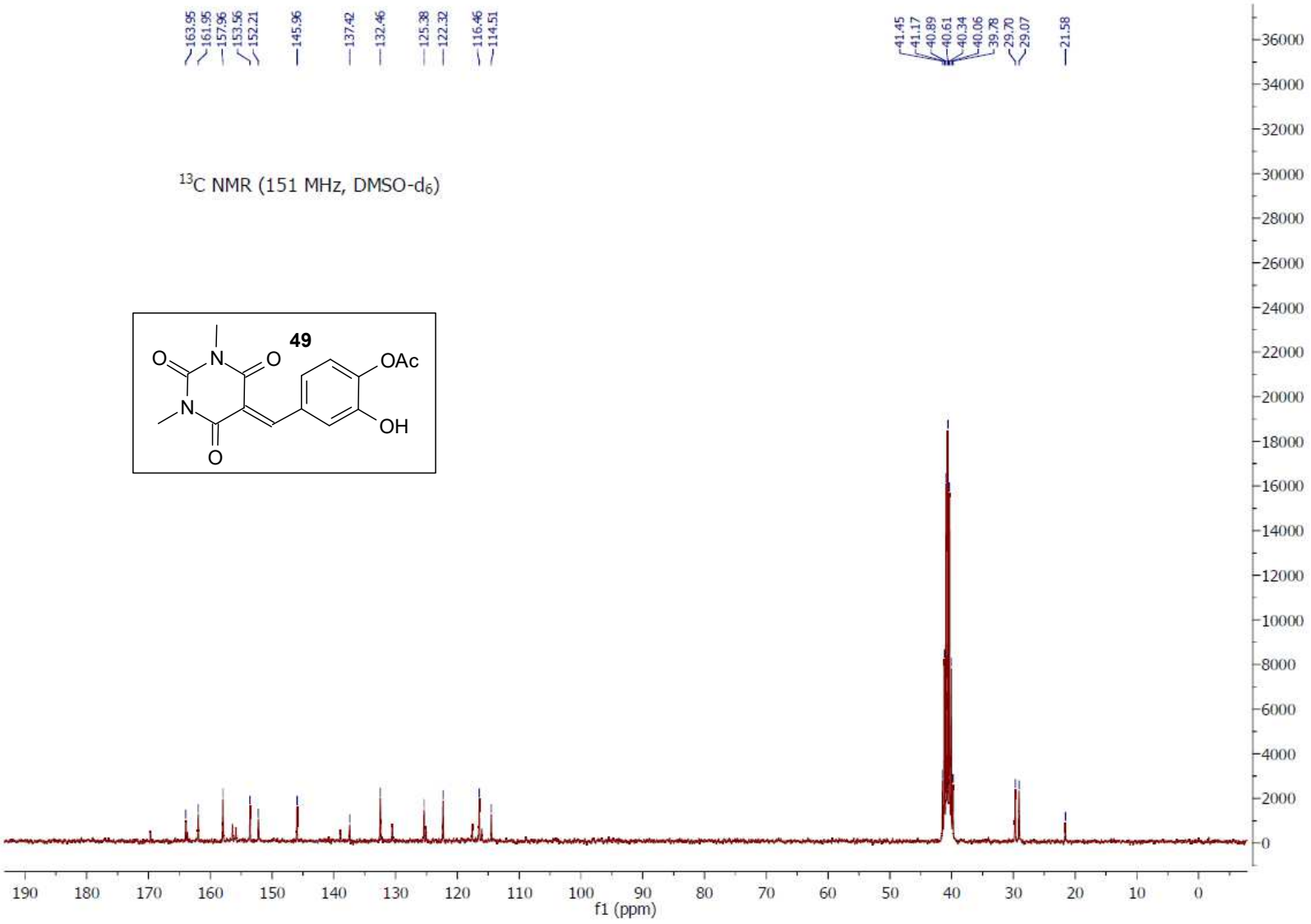
^{13}C NMR (151 MHz, DMSO-d_6




8.48
8.23
8.23
7.98
7.97
7.95
7.95
7.34
7.31
7.27
3.43
3.39
2.33
2.32
^{1}H NMR (300 MHz, $CDCl_3$)
44
O
N
O
N
O
OAc
OAc
1.00
0.99
1.01
1.00
5.99
6.02
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1 (ppm)


168.59
168.14
162.90
160.94
157.35
151.73
146.37
142.31
133.80
131.54
129.39
123.94
118.53
78.09
77.67
77.24
29.77
29.13
21.32
21.23
^{13}C NMR (151 MHz, $CDCl_3$)
44
OAc
OAc
f1 (ppm)


11.10
8.39
8.38
8.25
8.01
8.01
7.98
7.98
7.03
7.01
3.33
3.23
3.21
3.20
2.50
2.49
2.31
2.28
^{1}H NMR (300 MHz, DMSO-d$_6$)
49
O
N
O
OAc
N
OH
O
0.91
1.02
1.06
1.00
1.00
7.21
3.05
f1 (ppm)


163.95
161.95
157.96
153.56
152.21
145.96
137.42
132.46
125.38
122.32
116.46
114.51
41.45
41.17
40.89
40.61
40.34
40.06
39.78
29.70
29.07
21.58
13C NMR (151 MHz, DMSO-d6)
49
O
N
O
N
O
O
OAc
OH
36000
34000
32000
30000
28000
26000
24000
22000
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)