Supporting Information

Optimization of *O3*-Acyl Kojic Acid Derivatives as Potent and Selective Human Neutrophil Elastase Inhibitors

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1. General considerations

Melting points (mp) were recorded on a Kofler camera Bock Monoscope M and are uncorrected.

Proton and carbon nuclear magnetic resonance (1H and 13C NMR) spectra were recorded on a Bruker Avance 400 (400 and 100 MHz, respectively). All chemical shifts are quoted on the δ scale in ppm using residual solvent peaks as the internal standard. Coupling constants (*J*) are reported in Hz with the following splitting abbreviations: s = singlet, d = doublet, t = triplet, m = multiplet.

Elemental analysis were performed in a Flash 2000 CHNS-O analyzer (ThermoScientific, UK).

Thin layer chromatography (TLC) was carried out using Merck aluminum backed sheets coated with 60 F254 silica gel. Visualization of the silica plates was achieved using a UV lamp (λmax = 254 nm).

Flash chromatography was made using a Combi Flash RF-200 device from Teledyne Isco with RediSepbnormal-phase silica flash columns and using gradients of Hexane/EtOAc.

All reagents were purchased from Aldrich or AlfaAesar and used without further purification.

2. Chemistry

5-Hydroxy-2-hydroxymethyl-1-phenyl-pyridin-4-one (4). Intermediate **4** is described in the literature and was achieved as reported by Pirrung,[1] and was used directly after recrystalization.

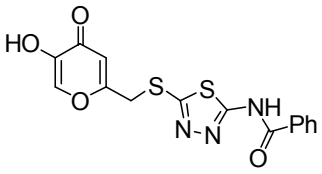
5-Hydroxy-2-hydroxymethyl-1- pyridone (5a) and 5-Hydroxy-2-chloromethyl-1-phenyl-pyridin-4-one (5b). Intermediates **5a** and **5b** were obtained via reaction with thionylchloride as described by Aytemir *et al*.[2]

2-mercaptobenzoxazole-5-carboxylic acid (6e). The building block **6e** is not commercially available and was obtained in agreement with Mulchande et al.[3]

General Procedure for the synthesis of intermediates 7a-i.

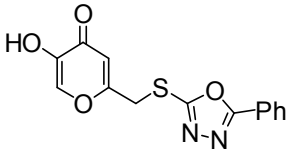
The thiol building blocks (**6a-e**) were dissolved in anydrous DMF (20 mL/mmol) and triethylamine was added (1.05 molar eq.) followed by the chorides **5a** or **5b** (1 molar eq.) and the reaction was left at room temperature overnight. The reaction mixture was poured into 10% HCl and the product extrated with EtOAc. Desired products were precipitated off with DCM/n-Hexane. Flash chromatography using n-Hexane/EtOAc gradient was performed when necessary.

N-(5-(((5-hydroxy-4-oxo-4H-pyran-2-yl)methyl)thio)-1,3,4-thiadiazol-2-yl)benzamide, 7a.



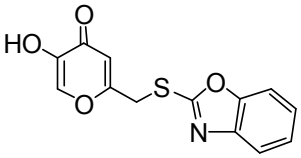
White solid; 95%; ^{1}H NMR (DMSO-*d6*, 400.13 MHz) δ 9.21 (1H, br s, N*H*); 8.10-8.12 (2H, m, Ar-*H*); 8.08 (1H, s, C*H*); 7.48-7.58 (3H, m, Ar-*H*); 6.37 (1H, s, C*H*); 4.36 (2H, s, CH_2).

5-hydroxy-2-(((5-phenyl-1,3,4-oxadiazol-2-yl)thio)methyl)-4H-pyran-4-one, 7b.



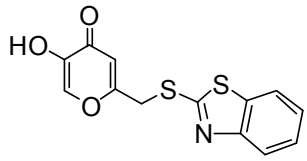
White solid; 82%; ^{1}H NMR (DMSO-*d6*, 400.13 MHz) δ 8.09 (1H, s, C*H*); 7.97-7.99 (2H, m, Ar-*H*); 7.58-7.65 (3H, m, Ar-*H*); 6.52 (1H, s, C*H*); 4.52 (2H, s, CH_2).

2-((benzo[d]oxazol-2-ylthio)methyl)-5-hydroxy-4H-pyran-4-one, 7c.



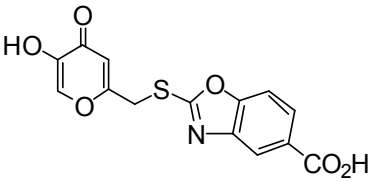
Pale yellow solid; 93%; ^{1}H NMR (DMSO-*d6*, 400.13 MHz) δ 8.08 (1H, s, C*H*); 8.05(1H, t, *J* = 7.7 Hz, Ar-*H*); 7.90(1H, t, *J* = 7.7 Hz, Ar-*H*); 7.50 (1H, t, *J* = 7.7 Hz, Ar-*H*); 7.40 (1H, t, , *J* = 7.7 Hz, Ar-*H*); 6.54 (1H, s, C*H*); 4.61 (2H, s, CH_2).

2-((benzo[d]oxazol-2-ylthio)methyl)-5-hydroxy-4H-pyran-4-one, 7d.



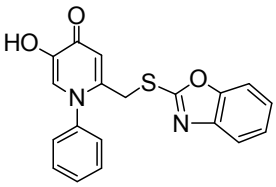
Pale yellow solid; 84%; ^{1}H NMR (DMSO-*d6*, 400.13 MHz) δ 7.23 (1H, s, C*H*); 7.19 (1H, d, *J* = 8.1 Hz, Ar-*H*); 7.04 (1H, d, *J* = 8.2 Hz, Ar-*H*); 6.64 (1H, t, *J* = 8.1 Hz, Ar-*H*); 6.54 (1H, t, , *J* = 8.1 Hz, Ar-*H*); 5.69 (1H, s, C*H*); 3.75 (2H, s, CH_2).

2-(((5-hydroxy-4-oxo-4H-pyran-2-yl)methyl)thio)benzo[d]thiazole-5-carboxylic acid, 7e.



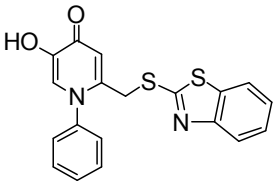
Pale yellow solid; 62%; ^{1}H NMR (400 MHz, DMSO) δ 13.13 (1H, s, CO2H), 9.22 (1H, s, OH), 8.16 (1H, s, CHarom), 8.08 (1H, s, CH), 7.97 (1H, d, *J* = 8.5 Hz, CHarom), 7.78 (1H, d, *J* = 8.5 Hz, CHarom), 6.58 (1H, s, CH), 4.59 (2H, s, CH2).

2-((benzo[d]oxazol-2-ylthio)methyl)-5-hydroxy-1-phenylpyridin-4(1H)-one, 7f.



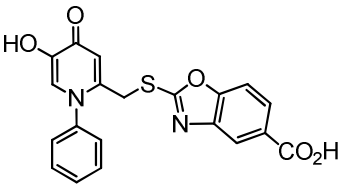
Pale yellow solid; 89%; ^{1}H NMR (DMSO-*d6*, 400.13 MHz) δ 7.45-7.63 (7H, m, Ar-*H*); 7.39 (1H, s, C*H*); 7.30-7.36 (2H, m, Ar-*H*); 6.57 (1H, s, C*H*); 4.34 (2H, s, CH_2).

2-((benzo[d]thiazol-2-ylthio)methyl)-5-hydroxy-1-phenylpyridin-4(1H)-one, 7g.



White solid; 76%; ^{1}H NMR (DMSO-*d6*, 400.13 MHz) δ 7.99 (1H, d, *J* = 7.6 Hz, Ar-*H*); 7.84 (1H, d, *J* = 8.1 Hz, Ar-*H*); 7.45-7.56 (6H, m, Ar-*H*); 7.39 (1H, s, C*H*); 7.37 (1H, t, *J* = 7.6 Hz, Ar-*H*); 6.56 (1H, s, C*H*); 4.37 (2H, s, CH_2).

2-((benzo[d]thiazol-2-ylthio)methyl)-5-hydroxy-1-phenylpyridin-4(1H)-one, 7h.

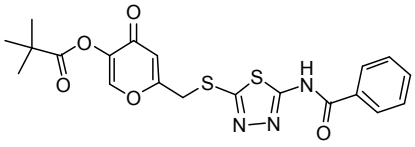


White solid; 76%; ^{1}H NMR (DMSO-*d6*, 400.13 MHz) δ 7.99 (1H, d, *J* = 7.6 Hz, Ar-*H*); 7.84 (1H, d, *J* = 8.1 Hz, Ar-*H*); 7.45-7.56 (6H, m, Ar-*H*); 7.39 (1H, s, C*H*); 7.37 (1H, t, *J* = 7.6 Hz, Ar-*H*); 6.56 (1H, s, C*H*); 4.37 (2H, s, CH_2).

General Procedure for the synthesis of final compouns 8a-q.

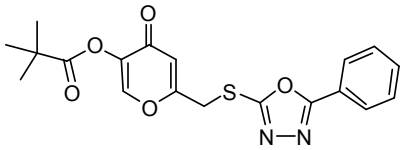
To a solution of intermediates **7a-h**, respectively, in DCM (0.05 M) was added TEA (1.2 eq.) and DMAP (cat.). The the corresponding acyl chloride was added (1.2 eq.) and the mixture was stirred at rt for 2-6h, as completion observed by TLC. The reaction mixture was poured into 10% HCl and the product extrated with DCM. Desired products were precipitated off with DCM/n-Hexane. Flash chromatography using n-Hexane/EtOAc gradient was performed when necessary.

6-(((5-benzamido-1,3,4-thiadiazol-2-yl)thio)methyl)-4-oxo-4H-pyran-3-yl pivalate, 8a.



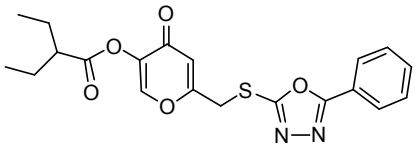
Pale yellow solid; 79%; mp (thermal decomposition at 225 ºC); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.19 (2H, d, *J* = 7.6 Hz, C*H*arom), 7.81 (1H, s, C*H*), 7.66 (1H, t, *J* = 7.6 Hz, C*H*arom), 7.56 (2H, t, *J* = 7.6 Hz, C*H*arom), 6.36 (1H, s, C*H*), 4.29 (2H, s, CH_2), 1.34 (9H, s, CH_3-Piv). ^{13}C+^{13}C -APT NMR (101 MHz, $CDCl_3$) 175.6 (Cq), 172.3 (Cq), 165.4 (Cq), 162.8 (Cq), 161.6 (Cq), 147.7 (CH), 141.4 (Cq), 133.5 (CH), 131.0 (Cq), 128.9 (2CH), 128.6 (2CH), 116.1 (CH), 39.2 (Cq), 34.0 (CH_2), 27.1 (CH_3). Anal. Calcd. ($C_{20}H_{19}N_3O_5S_2$): C, 53.92; H, 4.30; N, 9.43%. Found: C, 53.53; H, 4.27; N, 9.52%.

4-oxo-6-(((5-phenyl-1,3,4-oxadiazol-2-yl)thio)methyl)-4H-pyran-3-yl pivalate, 8b.



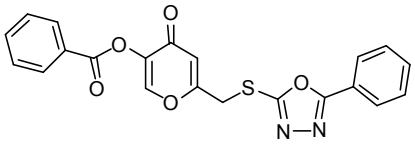
White solid; 65%; mp 130-131 ºC; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.01 (2H, m, C*H*arom), 7.85 (1H, s, C*H*), 7.54 (3H, m, C*H*arom), 6.66 (1H, s, C*H*), 4.37 (2H, s, CH_2), 1.36 (9H, s, CH_3-Piv). ^{13}C -APT NMR (101 MHz, $CDCl_3$) δ 175.5 (Cq), 172.2 (Cq), 166.5 (Cq), 162.1 (Cq), 161.9 (Cq), 147.7 (CH), 141.5 (Cq), 132.0 (CH), 129.1 (2CH), 126.8 (2CH), 123.2 (Cq), 116.4 (CH), 39.2 (Cq), 33.3 (CH_2), 27.1 (CH_3). Anal. Calcd. ($C_{19}H_{18}N_2O_5S$): C, 59.06; H, 4.70; N, 7.25%. Found: C, 59.58; H, 4.40; N, 7.45%.

4-oxo-6-(((5-phenyl-1,3,4-oxadiazol-2-yl)thio)methyl)-4H-pyran-3-yl 2-ethylbutanoate, 8c.



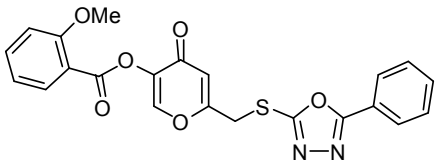
White solid; 79%; mp 83-84 ºC; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.03 – 8.00 (2H, m, CHarom), 7.86 (1H, s, CH), 7.59-7.98 (3H, m, CHarom), 6.67 (1H, s, CH), 4.38 (2H, s, CH_2), 2.53-2.46 (1H, m, CH), 1.79 – 1.66 (4H, m, 2CH_2), 1.02 (6H, t, *J* = 7.4 Hz, 2CH_3). ^{13}C -APT NMR (101 MHz, $CDCl_3$) δ 173.1 (Cq), 172.2 (Cq), 166.5 (Cq), 162.0 (Cq), 147.9 (CH), 141.2 (Cq), 132.0 (CH), 129.1 (2CH), 126.8 (2CH), 123.2 (Cq), 116.5 (CH), 48.4 (CH), 33.3 (CH_2), 24.9 (2CH_2), 11.7 (2CH_3). Anal. Calcd. ($C_{20}H_{20}N_2O_5S$): C, 59.99; H, 5.03; N, 7.00%. Found: C, 60.20; H, 5.10; N, 6.94%.

4-oxo-6-(((5-phenyl-1,3,4-oxadiazol-2-yl)thio)methyl)-4H-pyran-3-yl benzoate, 8d.



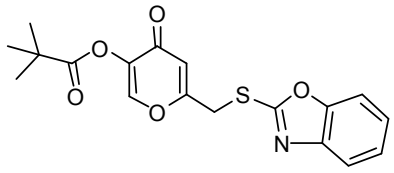
White solid; 98%; mp 149-150 ºC; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.18-8.02 (5H, m,CH, 4CHarom), 7.56-7.49 (6H, m, 6CHarom), 6.76 (1H, s, CH), 4.42 (2H, s,CH_2). ^{13}C -APT NMR (101 MHz, $CDCl_3$) δ 166.5 (Cq), 162.3 (2Cq), 148.2 (CH), 141.4 (Cq), 134.1 (CH), 132.0 (CH), 130.6 (CH), 129.1 (CH), 128.6 (CH), 126.8 (CH), 123.2 (Cq), 116.6 (CH), 33.3 (CH_2). Anal. Calcd. ($C_{21}H_{14}N_2O_5S.0.75H_2O$): C, 60.06; H, 3.73; N, 6.67%. Found: C, 60.07; H, 3.82; N, 6.14%.

4-oxo-6-(((5-phenyl-1,3,4-oxadiazol-2-yl)thio)methyl)-4H-pyran-3-yl 2-methoxybenzoate, 8e.



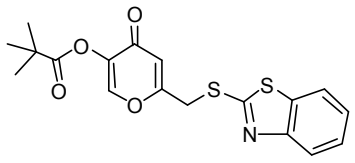
White solid; 96%; mp 140-141 ºC; ^{1}H NMR (400 MHz, $CDCl_3$) 8.07-7.99 (4H, m, CH, CHarom), 7.57 – 7.47 (4H, m, CHarom), 7.01 (2H, t, *J* = 8.1 Hz, CHarom), 6.70 (1H, s, CH), 4.38 (2H, s, CH_2), 3.91 (3H, s, OCH_3). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.3 (Cq), 166.5 (Cq), 162.2 (Cq), 162.1 (Cq), 161.9 (Cq), 160.3 (Cq), 148.3 (CH), 141.4 (Cq), 135.0 (CH), 132.9 (CH), 132.0 (CH), 129.1 (CH), 126.8 (CH), 123.3 (Cq), 120.3 (CH), 117.3 (CH), 116.5 (CH), 112.2 (CH), 56.1 (OCH3), 33.4 (CH2).Anal. Calcd. ($C_{22}H_{16}N_2O_6S$): C, 60.54; H, 3.70; N, 6.42%. Found: C, 60.53; H, 3.79; N, 6.04%.

6-((benzo[d]oxazol-2-ylthio)methyl)-4-oxo-4H-pyran-3-yl pivalate, 8f.



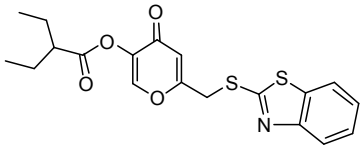
White solid; 81%; mp 137-138 ºC; ^{1}H NMR (400 MHz, $CDCl_3$) 7.84 (1H, s, CH), 7.62 (1H, d, *J* = 7.7 Hz, CHarom), 7.47 (1H, d, *J* = 7.7 Hz, CHarom), 7.36 – 7.24 (2H, m, CHarom), 6.69 (1H, s, CH), 4.38 (2H, s, CH2), 1.35 (9H, s, CH3-Piv). Anal. Calcd. ($C_{18}H_{17}NO_5S$): C, 60.15; H, 4.77; N, 3.90%. Found: C, 60.02; H, 4.79; N, 3.97%.

6-((benzo[d]thiazol-2-ylthio)methyl)-4-oxo-4H-pyran-3-yl pivalate, 8g.



White solid; 78%; mp 150-151 ºC; ^{1}H NMR (400 MHz, DMSO) δ 8.52 (1H, s, CH), 8.05 (1H, d, *J* = 7.9 Hz, CHarom), 7.90 (1H, d, *J* = 8.1 Hz, CHarom), 7.50 (2H, t, *J* = 7.7 Hz, 2CHarom), 7.41 (2H, t, *J* = 7.6 Hz, 2CHarom), 6.63 (1H, s, CH), 4.67 (2H, s, CH2), 1.25 (9H, s, CH_3-Piv). ^{13}C -APT NMR (101 MHz, $CDCl_3$) δ 181.8 (Cq), 175.3 (Cq), 172.0 (Cq), 164.65 (2Cq), 152.8 (Cq), 149.8 (CH), 141.0 (Cq), 135.4 (Cq), 127.0 (CH), 125.4 (CH), 122.5 (CH), 121.9 (CH), 115.8 (CH), 33.9 (CH_2), 27.24 (CH_3). Anal. Calcd. ($C_{18}H_{17}NO_4S_2$): C, 57.58; H, 4.56; N, 3.73%. Found: C, 57.99; H, 4.82; N, 3.67%.

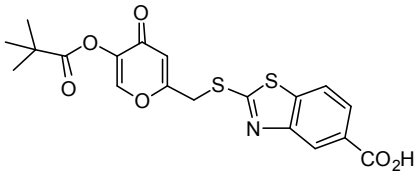
6-((benzo[d]thiazol-2-ylthio)methyl)-4-oxo-4H-pyran-3-yl 2-ethylbutanoate, 8h.



White solid; 78%; mp 93-94 ºC; ^{1}H NMR (400 MHz, DMSO) δ 7.91 (1H, d, *J* = 8.1 Hz, CHarom), 7.85 (1H, s, CH), 7.79 (1H, d, *J* = 8.0 Hz, CHarom), 7.46 (1H, t, *J* = 7.8 Hz, CHarom), 7.35 (1H, t, *J* = 7.8 Hz, CHarom), 6.67 (1H, s, CH), 4.48 (2H, s,

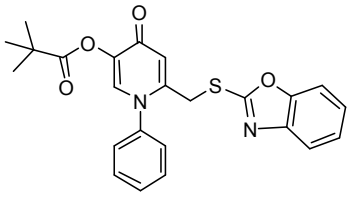
CH2), 2.51 (1H, m, CH), 1.80-1.64 (4H, m, 2 CH_2), 1.02 (6H, t, *J* = 7.4 Hz, CH_3). ^{13}C -APT NMR (101 MHz, $CDCl_3$) δ 173.1 (Cq), 172.5 (Cq), 163.3 (2Cq), 152.6 (Cq), 147.9 (CH), 141.1 (Cq), 135.4 (Cq), 126.3 (CH), 124.8 (CH), 121.9 (CH), 121.2 (CH), 116.3 (CH), 48.4 (CH), 33.6 (CH_2), 24.9 ($2CH_2$), 11.7 ($2CH_3$). Anal. Calcd. ($C_{19}H_{19}NO_4S_2$): C, 58.59; H, 4.92; N, 3.60%. Found: C, 59.16; H, 5.02; N, 3.63%.

2-(((4-oxo-5-(pivaloyloxy)-4H-pyran-2-yl)methyl)thio)benzo[d]thiazole-5-carboxylic acid, 8i.



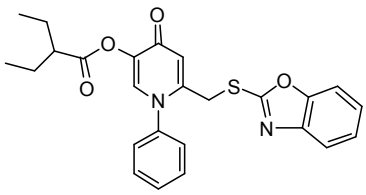
Pale yellow solid; 78%; mp (thermal decomposition at 207 ºC); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.44 (1H, s, CHarom), 8.12 (1H, d, *J* = 8.6 Hz, CHarom), 7.88 (1H, s, CH), 7.54 (1H, d, *J* = 8.6 Hz, CHarom), 6.82 (1H, s, CH), 4.42 (2H, s, CH_2), 1.36 (9H, s, CH_3). ^{13}C NMR (101 MHz, $CDCl_3$) δ 175.6 (Cq), 172.6 (Cq), 170.1 (Cq), 164.1 (Cq), 162.5 (Cq), 155.4 (Cq), 147.9 (CH), 141.6 (Cq), 141.4 (Cq), 127.0 (CH), 126.3 (Cq), 121.41 (CH), 116.4 (CH), 110.0 (CH), 39.2 (Cq), 33.1 (CH_2), 27.1 (CH_3). Anal. Calcd. ($C_{19}H_{17}NO_6S_2$.0.35n-Hex): C, 56.36; H, 4.92; N, 3.12%. Found: C, 56.28; H, 4.74; N, 3.45%.

6-((benzo[d]oxazol-2-ylthio)methyl)-4-oxo-1-phenyl-1,4-dihydropyridin-3-yl pivalate, 8j.



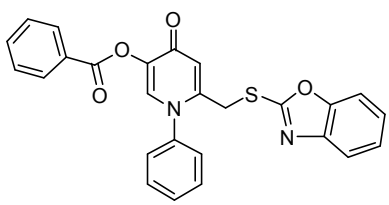
White solid; 77%; mp 188-189 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.58-7.41 (8H, m, 7CHarom+CH), 7.32-7.24 (2H, m, CHarom), 6.90 (1H, s, CH), 4.25 (2H, s, CH_2), 1.36 (9H, s, CH_3). ^{13}C-APT NMR (101 MHz, $CDCl_3$) δ 176.1 (Cq), 171.7 (Cq), 162.1 (Cq), 152.0 (Cq), 145.1 (Cq), 141.4 (Cq), 141.0 (Cq), 140.2 (Cq), 134.4 (CH), 130.3 (CH), 130.2 (CH), 127.5 (CH), 124.5 (CH), 124.3 (CH), 119.8 (CH), 118.7 (CH), 110.0 (CH), 39.2 (Cq), 32.8 (CH_2), 27.2 (CH_3). Anal. Calcd. ($C_{24}H_{22}N_2O_4S$): C, 66.34; H, 5.10; N, 6.45%. Found: C, 65.99; H, 5.11; N, 6.63%.

6-((benzo[d]oxazol-2-ylthio)methyl)-4-oxo-1-phenyl-1,4-dihydropyridin-3-yl 2-ethylbutanoate, 8k.



White solid; 85%; mp (thermal decomposition at 192 ºC). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.58-7.42 (8H, m, 7CHarom+CH), 7.32-7.25 (2H, m, CHarom), 6.92 (1H, s, CH), 4.25 (2H, s, CH_2), 2.50 (1H, m, CH), 1.83-1.63 (4H, m, $2CH_2$), 1.02 (6H, t, *J* = 7.4 Hz, $2CH_3$). ^{13}C-APT NMR (101 MHz, $CDCl_3$) δ 173.6 (Cq), 171.8 (Cq), 162.1 (Cq), 152.0 (Cq), 145.2 (Cq), 141.4 (Cq), 140.7 (Cq), 140.2 (Cq), 134.6 (CH), 130.3 (CH), 130.2 (CH), 127.5 (CH), 124.5 (CH), 124.3 (CH), 120.0 (CH), 118.7 (CH), 110.0 (CH), 48.5 (CH), 32.7 (CH_2), 24.9 ($2CH_2$), 11.8 ($2CH_3$). Anal. Calcd. ($C_{25}H_{24}N_2O_4S$): C, 66.94; H, 5.39; N, 6.25%. Found: C, 66.99; H, 5.40; N, 6.24%.

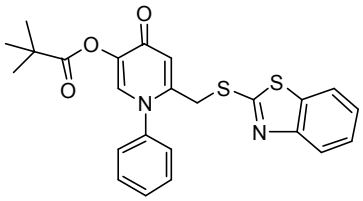
6-((benzo[d]oxazol-2-ylthio)methyl)-4-oxo-1-phenyl-1,4-dihydropyridin-3-yl benzoate, 8l.



Pale orange solid; 79%; mp (181-182 ºC). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.19 (2H, d, *J* = 7.9 Hz, CHarom), 7.64 (1H, s, CH), 7.62-7.43 (10H, m, CHarom), 7.31-7.23 (2H, m, CHarom), 7.01 (1H, s, CH), 4.29 (2H, s, CH_2).). ^{13}C-APT

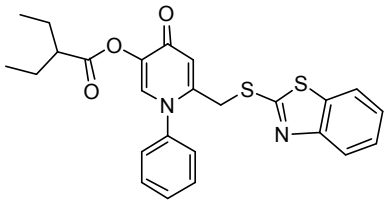
NMR (101 MHz, $CDCl_3$) δ 171.9 (Cq), 164.1 (Cq), 152.1 (Cq), 145.3 (Cq), 141.4 (Cq), 140.9 (Cq), 140.2 (Cq), 134.9 (CH), 133.7 (CH), 130.5 (CH), 130.4 (CH), 130.3 (CH), 128.7 (Cq), 128.5 (CH), 127.5 (CH), 124.6 (CH), 124.4 (CH), 120.1 (CH), 118.8 (CH), 110.1 (CH), 32.79 (CH_2). Anal. Calcd. ($C_{26}H_{18}N_2O_4S$): C, 68.71; H, 3.99; N, 6.16%. Found: C, 68.22; H, 4.06; N, 6.22%.

6-((benzo[d]thiazol-2-ylthio)methyl)-4-oxo-1-phenyl-1,4-dihydropyridin-3-yl pivalate, 8m.



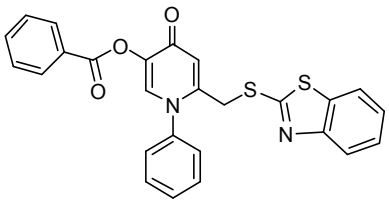
White solid; 93%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.82 (1H, d, *J* = 8.1 Hz, CHarom), 7.74 (1H, d, *J* = 8.0 Hz, CHarom), 7.54-7.40 (7H, m, 6CHarom+CH), 7.32 (1H, t, *J* = 7.6 Hz, CHarom), 6.83 (1H, s, CH), 4.33 (2H, s, CH_2), 1.36 (9H, s, $3CH_3$). ^{13}C-APT NMR (101 MHz, $CDCl_3$) δ 171.8 (Cq), 171.4 (Cq), 163.4 (Cq), 152.5 (Cq), 147.0 (Cq), 145.6 (Cq), 144.1 (Cq), 140.9 (Cq), 140.3 (Cq), 135.4 (Cq), 134.29, 130.1 (CH), 127.5 (CH), 127.2 (CH), 126.2 (CH), 126.2 (CH), 124.7 (CH), 124.7 (CH), 123.0 (CH), 121.8 (CH), 121.1 (CH), 121.1 (CH), 119.9 (CH), 114.0 (CH), 39.2 (Cq), 33.4 (CH_2), 27.3 ($3CH_3$). Anal. Calcd. ($C_{24}H_{22}N_2O_3S_2$): C, 63.98; H, 4.92; N, 6.22%. Found: C, 63.32; H, 5.37; N, 6.67%.

6-((benzo[d]thiazol-2-ylthio)methyl)-4-oxo-1-phenyl-1,4-dihydropyridin-3-yl 2-ethylbutanoate, 8n.



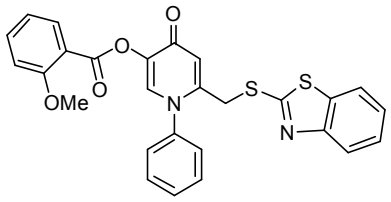
White solid; 79%; mp (64-65 ºC). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.83 (1H, d, *J* = 8.1 Hz, CHarom), 7.74 (1H, d, *J* = 8.0 Hz, CHarom), 7.55-7.39 (7H, m, 6CHarom+CH), 7.32 (1H, t, *J* = 7.8 Hz, CHarom), 6.85 (1H, s, CH), 4.33 (2H, s, CH2), 2.54-2.47 (1H, m, CH), 1.86-1.74 (2H, m, CH2), 1.70-1.59 (2H, m, CH2), 1.03 (1H, t, *J* = 7.4 Hz, CH3). ^{13}C-APT NMR (101 MHz, $CDCl_3$) δ 171.9 (Cq), 164.1 (Cq), 152.1 (Cq), 145.3 (Cq), 141.4 (Cq), 140.9 (Cq), 140.2 (Cq), 134.9 (CH), 133.7 (CH), 130.5 (CH), 130.4 (CH), 130.3 (CH), 128.7 (Cq), 128.5 (CH), 127.5 (CH), 124.6 (CH), 124.4 (CH), 120.1 (CH), 118.8 (CH), 110.1 (CH), 32.79 (CH_2). Anal. Calcd. ($C_{25}H_{24}N_2O_3S_2$): C, 64.63; H, 5.21; N, 6.03%. Found: C, 64.19; H, 5.16; N, 6.10%.

6-((benzo[d]thiazol-2-ylthio)methyl)-4-oxo-1-phenyl-1,4-dihydropyridin-3-yl benzoate, 8o.



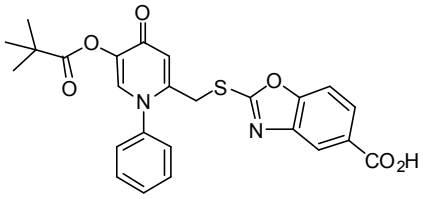
White solid; 82%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.19 (2H, d, *J* = 7.6 Hz, 2CHarom), 7.85 (1H, d, *J* = 8.1 Hz, CHarom), 7.75 (1H, d, *J* = 8.0 Hz, CHarom), 7.66-7.58 (2H, m, CHarom+CH), 7.55-7.41 (8H, m, CHarom), 7.33 (1H t, *J* = 7.7 Hz, CHarom), 6.96 (1H, s, CH), 4.38 (2H, s, CH_2). ^{13}C-APT NMR (101 MHz, $CDCl_3$) δ 171.8 (Cq), 164.1 (Cq), 163.4 (Cq), 152.5 (Cq), 146.0 (Cq), 140.7 (Cq), 140.3 (Cq), 135.4 (Cq), 134.8 (CH), 133.7 (CH), 130.5 (CH), 130.3 (CH), 130.2 (CH), 128.7 (Cq), 128.5 (CH), 127.4 (CH), 126.2 (CH), 124.7 (CH), 121.8 (CH), 121.1 (CH), 120.0 (CH), 33.2 (CH_2). Anal. Calcd. ($C_{26}H_{18}N_2O_3S_2$): C, 66.36; H, 3.86; N, 5.95%. Found: C, 66.68; H, 3.81; N, 5.94%.

6-((benzo[d]thiazol-2-ylthio)methyl)-4-oxo-1-phenyl-1,4-dihydropyridin-3-yl 2-methoxybenzoate, 8p.



White solid; 74%; mp (96-97 ºC). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.13 (1H, d, *J* = 7.8 Hz, CHarom), 7.84 (1H, d, *J* = 8.1 Hz, CHarom), 7.75 (1H, d, *J* = 7.9 Hz, CHarom), 7.65 (1H, s, CH), 7.58-7.38 (7H, m, CHarom), 7.32 (1H, t, *J* = 7.6 Hz, CHarom), 7.02 (2H, m, CHarom), 6.93 (1H, s, CH), 4.36 (2H, s, CH_2), 3.91 (3H, s, OCH_3).^{13}C NMR (101 MHz, $CDCl_3$) δ 172.0 (Cq), 163.4 (Cq), 163.0 (Cq), 160.1 (Cq), 152.5 (Cq), 145.7 (Cq), 140.6 (Cq), 140.3 (Cq), 135.4 (CH), 135.0 (CH), 134.6 (CH), 133.0 (CH), 130.2 (CH), 130.1 (CH), 127.5 (CH), 126.2 (CH), 124.7 (CH), 121.8 (CH), 121.1 (CH), 120.2 (CH), 119.9 (CH), 118.1 (Cq), 112.1 (CH), 56.1 (OCH_3), 33.3 (CH_2).

2-(((4-oxo-1-phenyl-5-(pivaloyloxy)-1,4-dihydropyridin-2-yl)methyl)thio)benzo[d]oxazole-5-carboxylic acid, 8q.



Pale yellow solid; 56%; ^{1}H NMR (400 MHz, DMSO) δ 8.11-7.94 (3H, m, CHarom+CH), 7.73-7.44 (7H, m, CHarom), 6.68 (1H, s, CH), 4.41 (2H, s, CH2), 1.24 (9H, s, CH3).^{13}C NMR (101 MHz, DMSO) δ 175.5 (Cq), 170.9 (Cq), 167.3 (Cq), 164.6 (Cq), 154.5 (Cq), 146.1 (Cq), 141.5 (Cq), 140.5 (Cq), 140.4 (Cq), 136.0 (CH), 130.3 (CH), 128.2 (CH), 128.1 (CH), 126.6 (CH), 119.8 (CH), 118.9 (CH) , 110.9 (CH), 32.9 (Cq), 27.4 (CH_3). Anal. Calcd. ($C_{25}H_{22}N_2O_6S$): C, 62.75; H, 4.63; N, 5.85 %. Found: C, 62.23; H, 5.03; N, 6.22%.

3. Molecular Docking

The 3D structure coordinates of HNE were obtained from the Protein Data Bank, PDB code 1HNE with X-ray coordinates at 1.84 Å resolution (previously validated for docking procedures[4]). To prepare the enzyme for the docking studies, the co-crystallized inhibitor as well as crystallographic waters, included in the PDB structure, were removed. Hydrogen atoms were added and the protonation states were correctly assigned using the Protonate-3D tool within the Molecular Operating Environment (MOE) 2012.10 software package [5], energy was minimized using MMFF94x forcefield. Molecular docking studies were then performed using the GoldScore scoring function from GOLD software package[6] and each ligand was subjected to 500 docking runs.

4. Pharmacological Methods

For all serine proteases (human neutrophil elastase, porcine pancreatic elastase, cathepsin G, proteinase 3, thrombin, kallikrein, urokinase, trypsin and chymotrypsin), activity was monitored for 30 min at excitation and emission wavelengths of 360 and 460 nm, respectively in a microplate reader (FLUOstar Omega, BMG Labtech, Germany). For all compounds tested,

the concentration of inhibitor that caused 50% inhibition of the enzymatic reaction (IC_{50}) was determined by non-linear regression using GraphPad PRISM software. Inhibitors stock solutions were prepared in DMSO, and serial dilutions were made in DMSO. Assays were performed in triplicate and data presented as the mean and the standard deviation.

4.1 Inhibition Assay for human neutrophil elastase

Fluorometric assays for the human neutrophil elastase (HNE) (Merck, Germany) inhibition activity were carried out in 200 µL assay buffer (0.1 M HEPES pH 7.5 at 25 °C) containing 20 µL of 0.17 µM HNE in assay buffer (stock solution 1.7 µM in 0.05 M acetate buffer, pH 5.5), 155 µL of assay buffer and 5 µL of each concentration of tested inhibitors. After 30 min of incubation at 25ºC the reaction was initiated by the addition of 20 µl of fluorogenic substrate to final concentration 200 µM (MeO-Suc-Ala-Ala-Pro-Val-AMC, Merck, Germany). The *K*m of this substrate of HNE was previously determined to be 185 µM (data not shown). For all assays, saturated substrate concentration was used, throughout, in order to obtain linear fluorescence curves. Controls were performed using enzyme alone, substrate alone, enzyme with DMSO and a positive control (Sivelestat sodium salt hydrate, Sigma Aldrich, UK).

4.2 Inhibition Assay for porcine pancreatic elastase

The inhibition of PPE was assayed by incubation method, 5 µl of inhibitor solution in DMSO was incubated at 25 ºC with 155 µL of 0.1 M HEPES buffer, pH 7.5, and 20 µL of PPE solution (50 µM in 0.1 M HEPES buffer, pH 7.5). After 30min of incubation at 25ºC the reaction was initiated by the addition of 20 µL of fluorogenic substrate to final concentration of 200 µM (MeO-Suc-Ala-Ala-Pro-Val-AMC, Merck, Germany) and activity was monitored (excitation 380 nm; emission 460 nm) for 30 min, at 25ºC on a Fluorescence Microplate Reader Tecan infinite M200 (Tecan, Switzerland). Inhibitors stock solutions were prepared in DMSO, and serial dilutions were made in DMSO. Controls were performed using enzyme alone, substrate alone and enzyme with DMSO and positive control (Elastase Inhibitor I, # 324692, CalBiochem).

4.3 Inhibition Assay for cathepsin G

Inactivation of cathepsin G (Calbiochem cat # 219373) was studied at 25 ºC using the progress curve method. A Chromogenic 96 well microplate assay for the Cathepsin G, Human Neutrophil (Calbiochem, Germany) inhibition activity was carried out in 200 µL assay buffer (0.1 M HEPES pH 7.5 at 25 °C) containing 5 µL of each concentration of tested inhibitors, 125 µL of assay buffer and 20 µL of 680 nM Cathepsin G (680 nM in 0.05 M acetate buffer, pH 5.5). After a period of 30 minutes of incubation at 25ºC the reaction was initiated by the addition of 50 µL of 3.4 mM chromogenic substrate (Suc-Ala-Ala-Pro-Phe-p-nitroanilide, Calbiochem, Germany) in assay buffer (stock solution 42.5 mM in DMSO). Controls were performed using enzyme alone, substrate alone, enzyme with DMSO and a positive control (Cathepsine G inhibitor I, Calbiochem, Germany).

4.4 Inhibition Assay for proteinase 3

Inactivation of proteinase 3 (Calbiochem cat #539483) was studied at 25 ºC in 200 µL assay buffer (0.1 M HEPES pH 7.5 at 25 °C) containing 70 µL of 65 nM proteinase 3 in assay buffer (stock solution 650 nM in 0.05 M acetate buffer, 150 mM NaCl, pH 5.5), 50 µL assay buffer and 5 µL of each concentration of tested inhibitors. The reaction was initiated by the addition of 75 µL of 10 mM chromogenic substrate (N-MeOSuc-Ala-Ala-Pro-Val-p-nitroanilide, stock solution

50 mM in DMSO, Sigma, UK) in assay buffer. Controls were performed using enzyme alone, substrate alone, enzyme with DMSO and a positive control (3,4-Dichloroisocoumarin, Calbiochem, Germany).

4.5 Inhibition Assay for thrombin

Inactivation of thrombin (Calbiochem) was studied at 25 ºC in reaction mixtures containing 0.01 M sodium phosphate, 0.138 M NaCl, 0.1% PEG 6000, pH 7.0, 1.7 U/mL human plasma thrombin, test compounds, and 50 µM substrate (Z-Gly-Gly-Arg-AMC.HCl, Bachem). Controls were performed using enzyme alone, substrate alone, enzyme with DMSO and a positive control (3,4-Dichloroisocoumarin, Calbiochem, Germany).

4.6 Inhibition Assay for kallikrein

The analysis of kallikrein (Calbiochem) inhibition was performed in reaction mixtures containing 0.05 M Tris-HCl, 0.138 M NaCl, pH 8.0, 2 nM human plasma kallikrein, test compounds, and 50 µM substrate (H-Pro-Phe-Arg-AMC acetate salt, Bachem). Controls were performed using enzyme alone, substrate alone, enzyme with DMSO and a positive control (Gabexate mesylate, Aldrich).

4.7 Inhibition Assay for urokinase

The analysis of urokinase (Calbiochem) inhibition assay was performed in reaction mixtures containing 0.05 M Tris-HCl, 0.138 M NaCl, pH 8.0, 30 U/mL human urine urokinase, test compounds, and 50 µM substrate (Z-Gly-Gly-Arg-AMC.HCl). Controls were performed using enzyme alone, substrate alone, enzyme with DMSO and a positive control.

4.8 Inhibition Assay for trypsin

The analysis of trypsin (Calbiochem) inhibition was performed in reaction mixtures containing 0.05 M Tris-HCl, 0.138 M NaCl, pH 8.0, 30nM human pancreas Trypsin, test compounds, and 50 µM substrate (Z-Gly-Gly-Arg-AMC.HCl, Bachem). Controls were performed using enzyme alone, substrate alone, enzyme with DMSO and a positive control (3,4-Dichloroisocoumarin, Calbiochem, Germany).

4.9 Inhibition Assay for chymotrypsin

Inactivation of chymotrypsin (Calbiochem) was studied at 25 ºC in reaction mixtures containing 0.05 M Tris-HCl, 0.138 M NaCl, pH 8.0, 30 nM human pancreas chymotrypsin, test compounds, and 100 µM substrate (Suc-Ala-Ala-Pro-Phe-7-amino-4-methylcoumarin, Bachem). Controls were performed using enzyme alone, substrate alone, enzyme with DMSO and a positive control (Gabexate mesylate, Aldrich).

4.10 In Vitro Cytotoxicity

The cytotoxicity was assessed using general cell viability endpoint MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide). Briefly, the day before experiment cells NIH 3T3 (mouse embryonic fibroblast cell line, ATCC CRL-1658) or HEK 293T (human embryonic kidney epithelial cell line, ATCC CRL-11268) were seeded in 96 well tissue culture plates, in RPMI 1640 culture medium supplemented with 10% Fetal serum bovine, 100 units of penicillin G (sodium salt) and 100µg of streptomycin sulfate and 2mM L-glutamine, at a concentration that allow cells to grow exponentially during the time of the assay. Compounds to be tested

were diluted in dimethylsulfoxide (DMSO) and then serially diluted in the culture medium. Compounds at different concentrations and DMSO were then added to the cells. Cells were incubated at 37°C in humidified 5% CO_2 atmosphere. After 48 hours, cell media containing DMSO (for control cells) or tested compound solution (for test cells) was removed and replaced with fresh medium containing MTT dye. After 3h of incubation the complete media was removed and the intracellular formazan crystals were solubilised and extracted with DMSO. After 15 min at room temperature the absorbance measured at 570 nm in microplate reader.

The percentage of cell viability was determined for each concentration of tested compound and the concentration of a compound reflecting a 50 % inhibition of cell viability (i.e. IC50) was determined from the concentration-response curve. This was done by applying non-linear regression procedure to the concentration response data using GraphPad PRISM software.

4.11 Chemical stability at pH 7.4

Chemical stability was determined for solutions of synthesized compounds (100 µM) in phosphate buffer (pH 7.4). Aliquots were taken in regular times and analysed by HPLC.

4.12 Stability in human plasma

Human plasma was obtained from the pooled, heparinised blood of healthy donors, and was frozen and stored at -20ºC prior to use. For the stability assay, the compounds (10 µL of a 10^{-2} M inhibitor stock solution), were incubated at 37 ºC in human plasma that had been diluted to 80% (v/v) with phosphate buffer pH 7.4. Aliquots were taken in regular times, the reaction was stopped by addition of MeCN and the samples were vortexed and centrifugated for 10 min and analysed by HPLC.

4.13 Stability toward microsomal activity

Stability was assayed against Rat Pooled Liver Microsomes Male (Sprague-Dawley) 20 mg/mL from BD Gentest™. A typical incubation medium was prepared, containing 10 µL of microsomal protein, 285 µL of H_2O, 80 µL phosphate buffer (pH 7.4) and NADPH generating system [20 µL of solution A ($NADP^+$ and G6P) and 4 µL of solution B (G6PDH), both from BD Gentest™], in a 37 ºC thermostatic bath. Reaction was started by addition of the test compounds (10 µL of a 10^{-3} M inhibitors stock solution). Aliquots were taken in regular times, the reaction was stopped by addition of MeCN and the samples were vortexed and centrifugated for 10 min and analysed by HPLC.

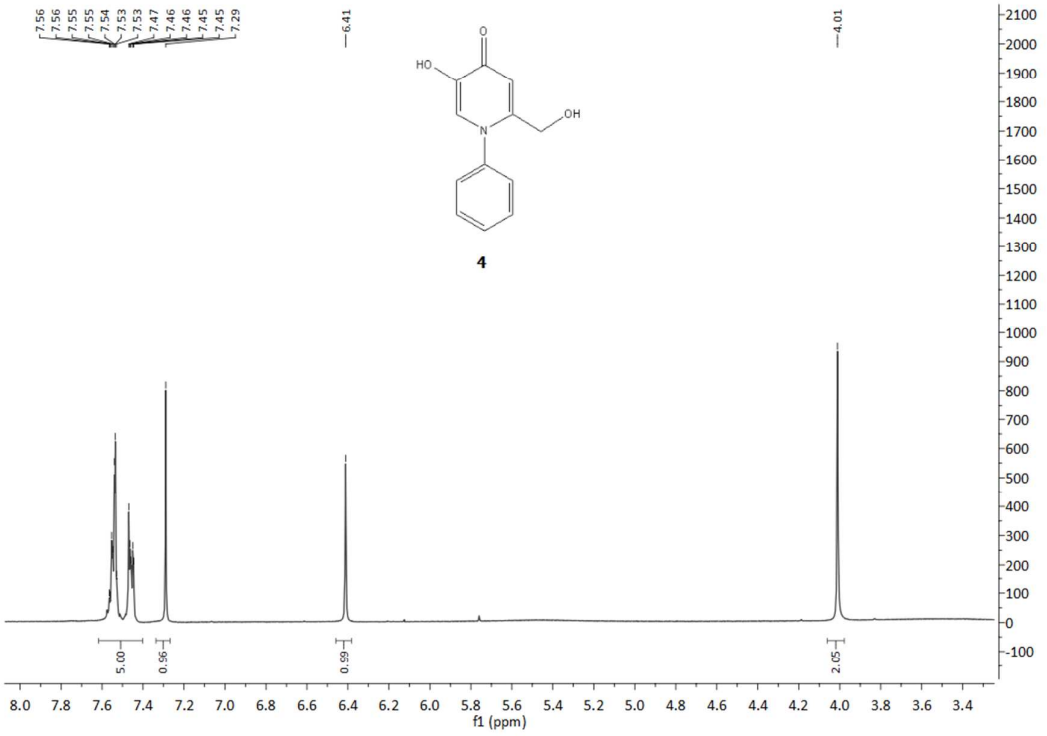
4.14 HPLC system

Merck Hitachi Pump L-2130; Column Oven L-2300; UV detector L-2400. Column LiChroCart Purospher RP-18 (5 μm, 250-4 mm). Gradient with H_2O/MeCN (90/10 to 15/85, v/v in 15 min) was used as mobile phase (1mL/min); 20 µL injection volumes.

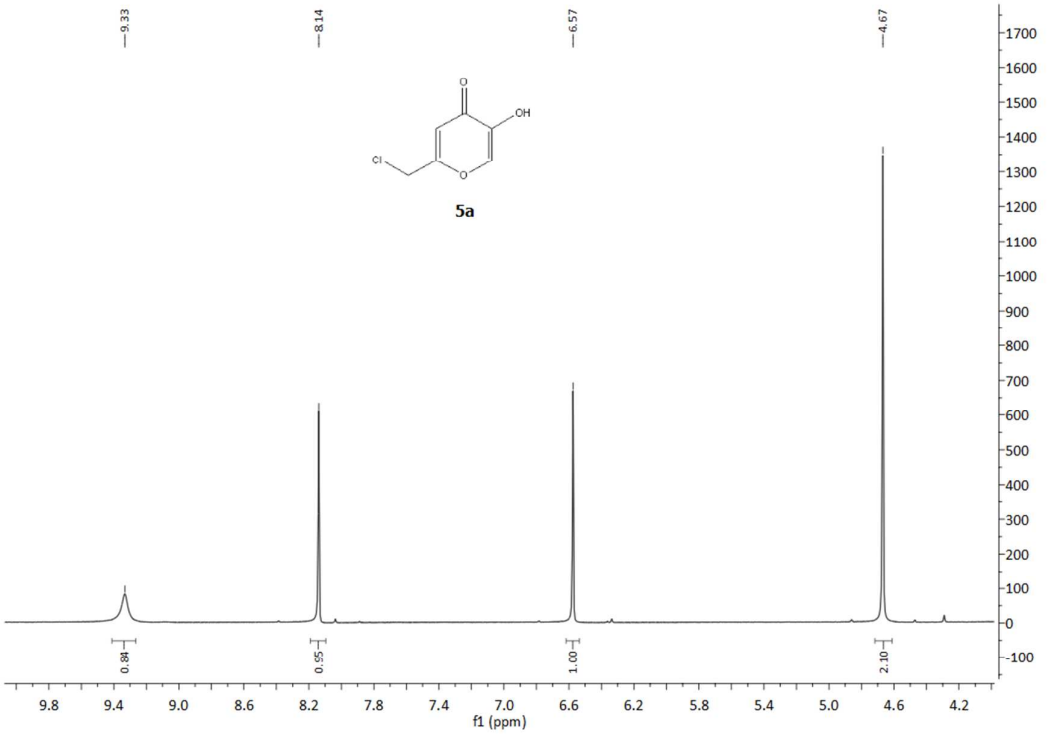
5. Suplemental Data

5.1Spectroscopical data for intermediates 4, 5 and 7.

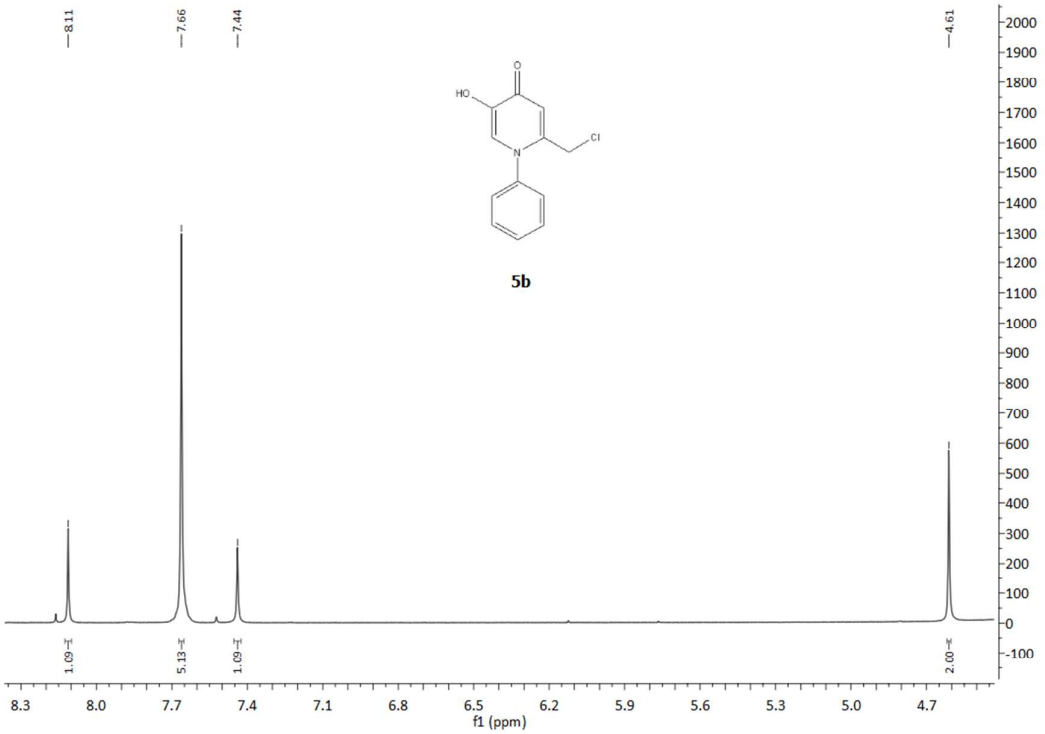
^{1}H-NMR(DMSO) for compound 4.



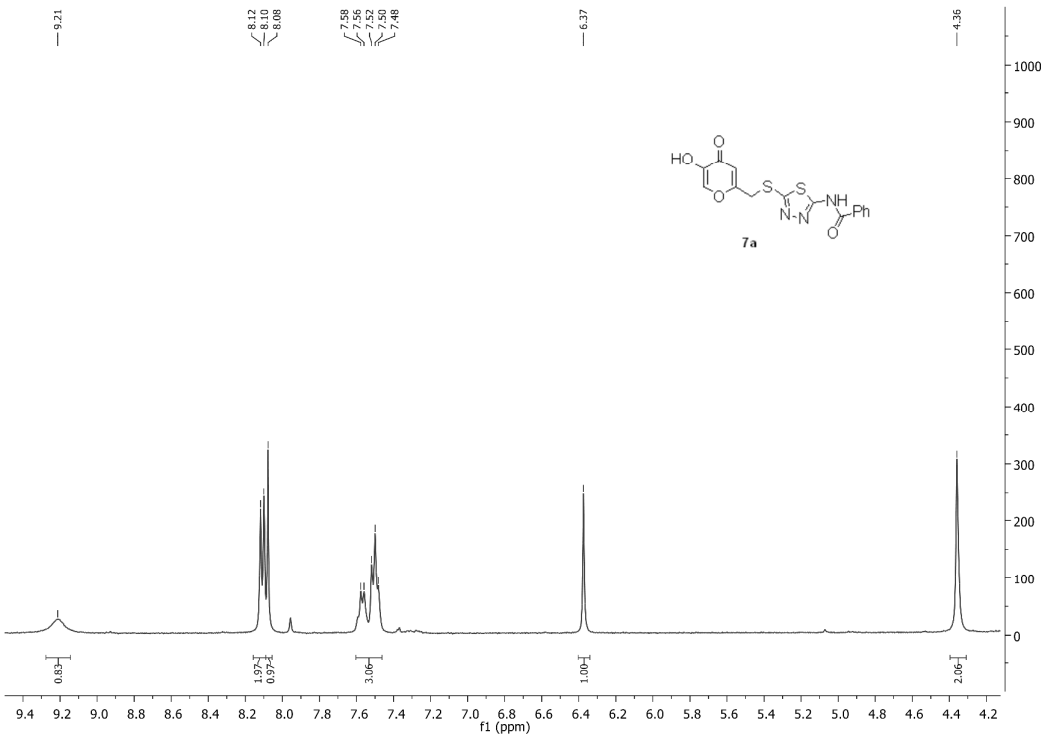
1H-NMR(DMSO) for compound 5a.



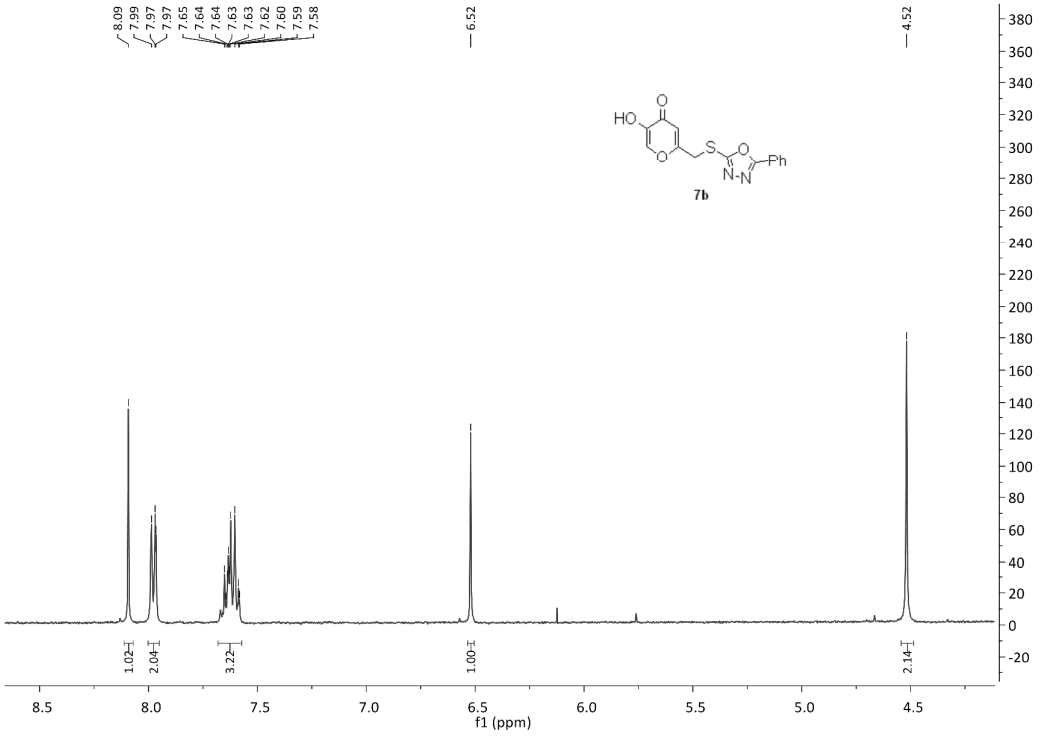
1H-NMR(DMSO) for compound 5b.



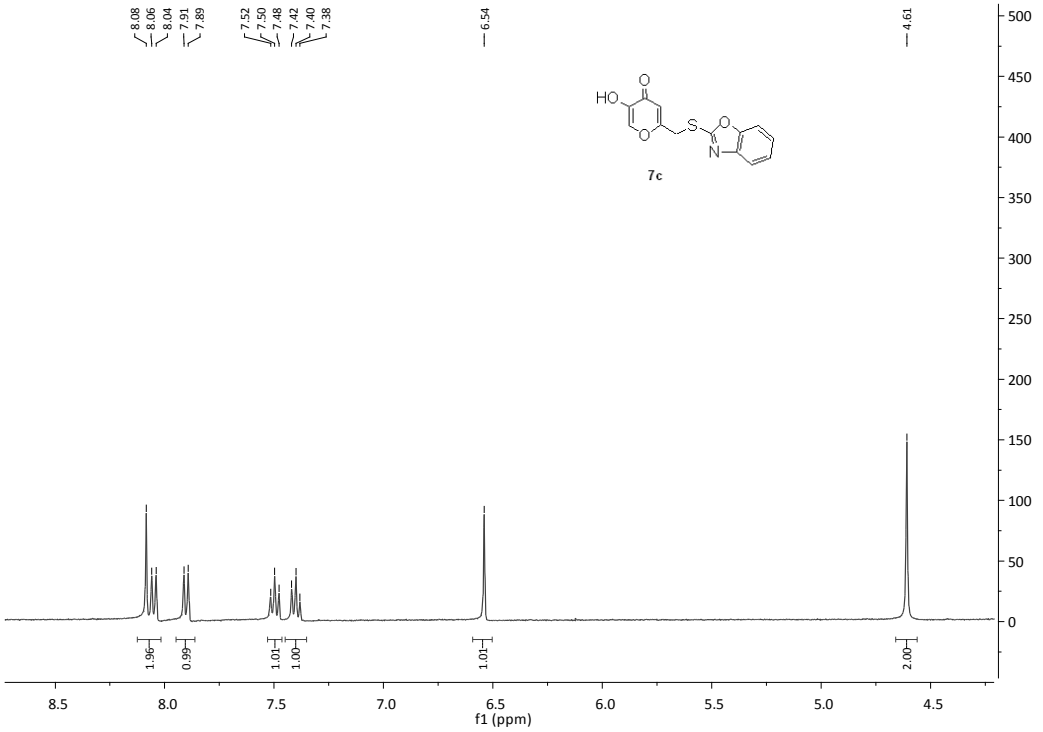
^{1}H-NMR(DMSO) for compound 7a.



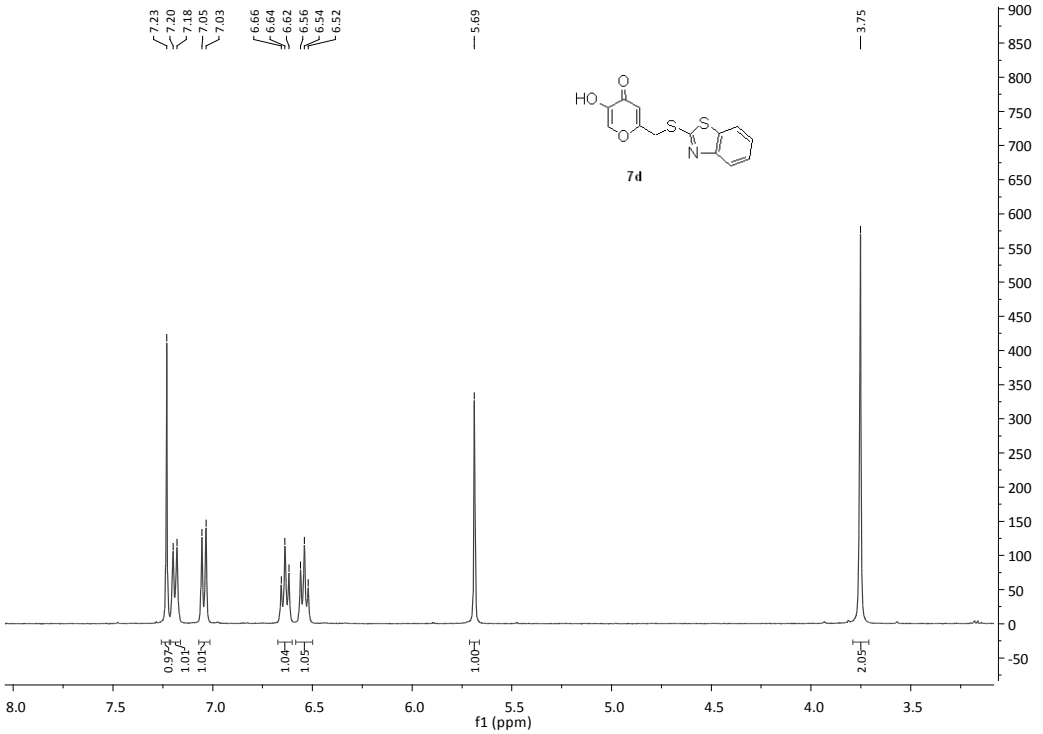
^{1}H-NMR(DMSO) for compound 7b.



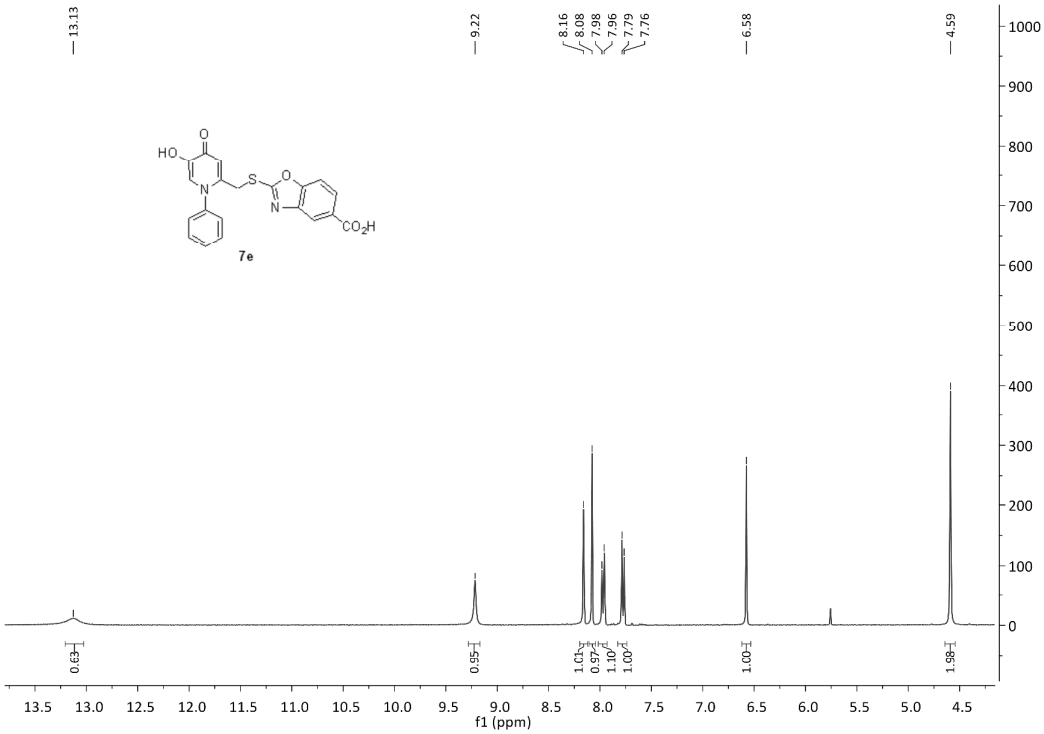
^{1}H-NMR(DMSO) for compound 7c.



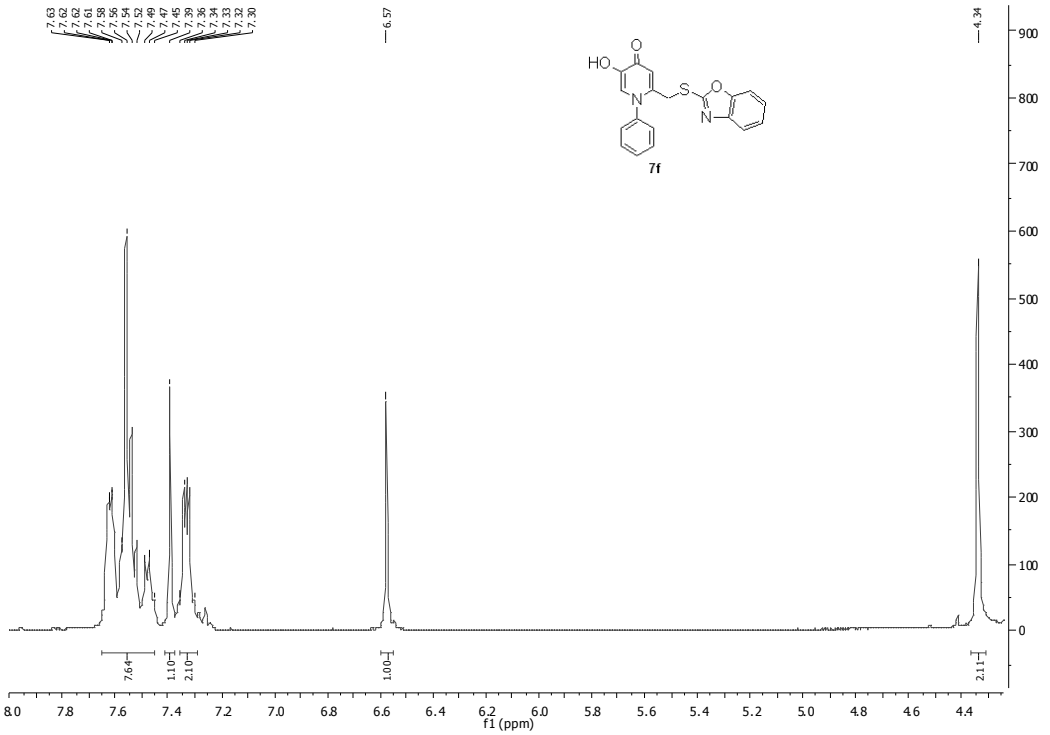
^{1}H-NMR(DMSO) for compound 7d.



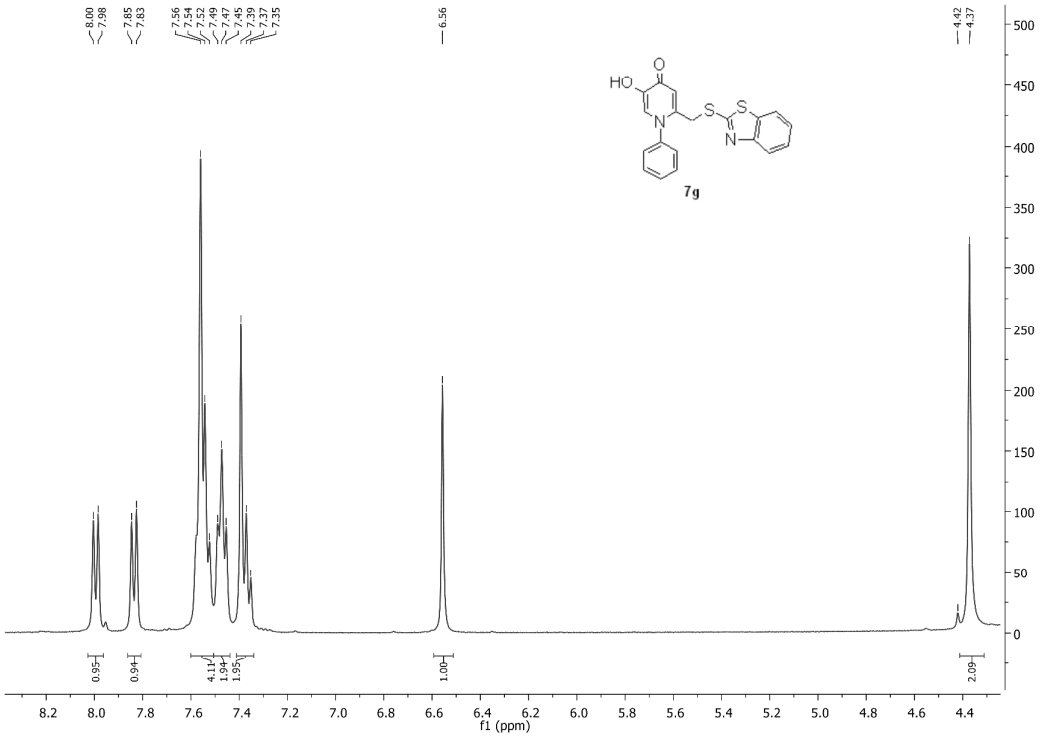
^{1}H-NMR(DMSO) for compound 7e.



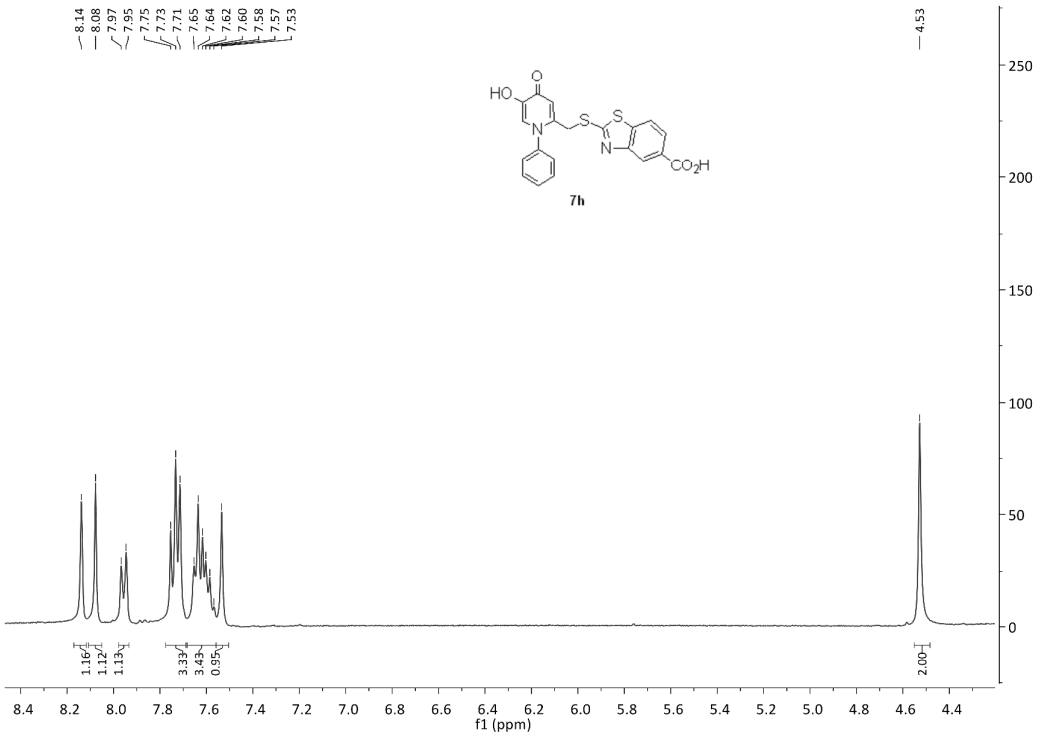
[1]H-NMR(DMSO) for compound 7f.



[1]H-NMR(DMSO) for compound 7g.

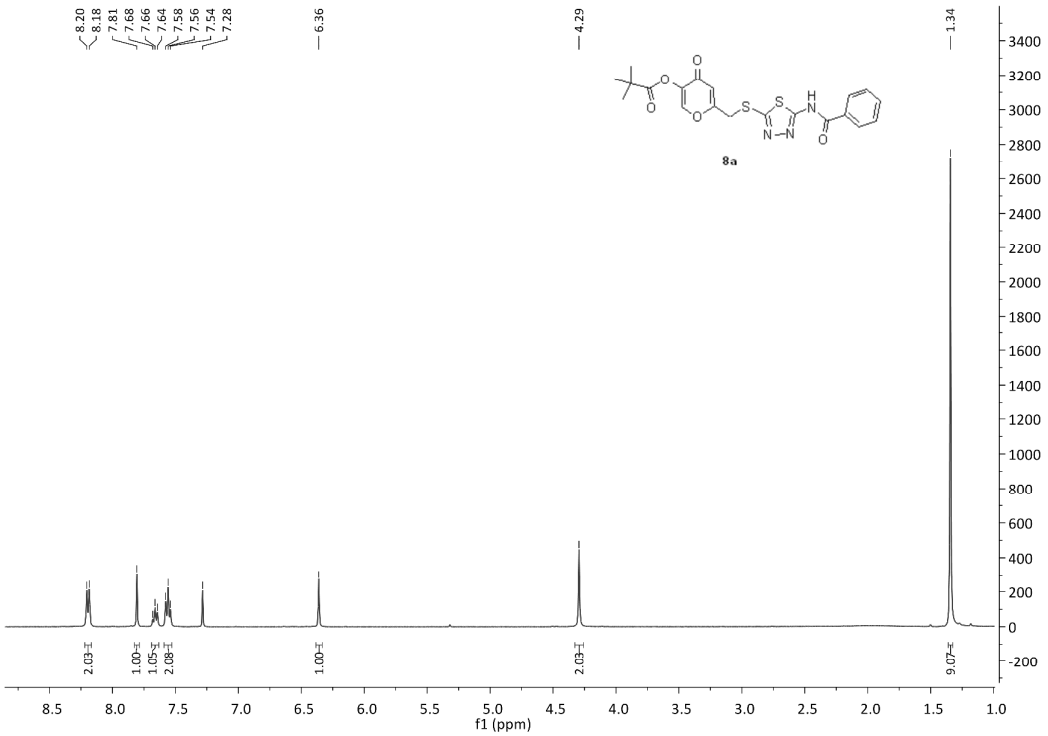


^{1}H-NMR(DMSO) for compound 7h.

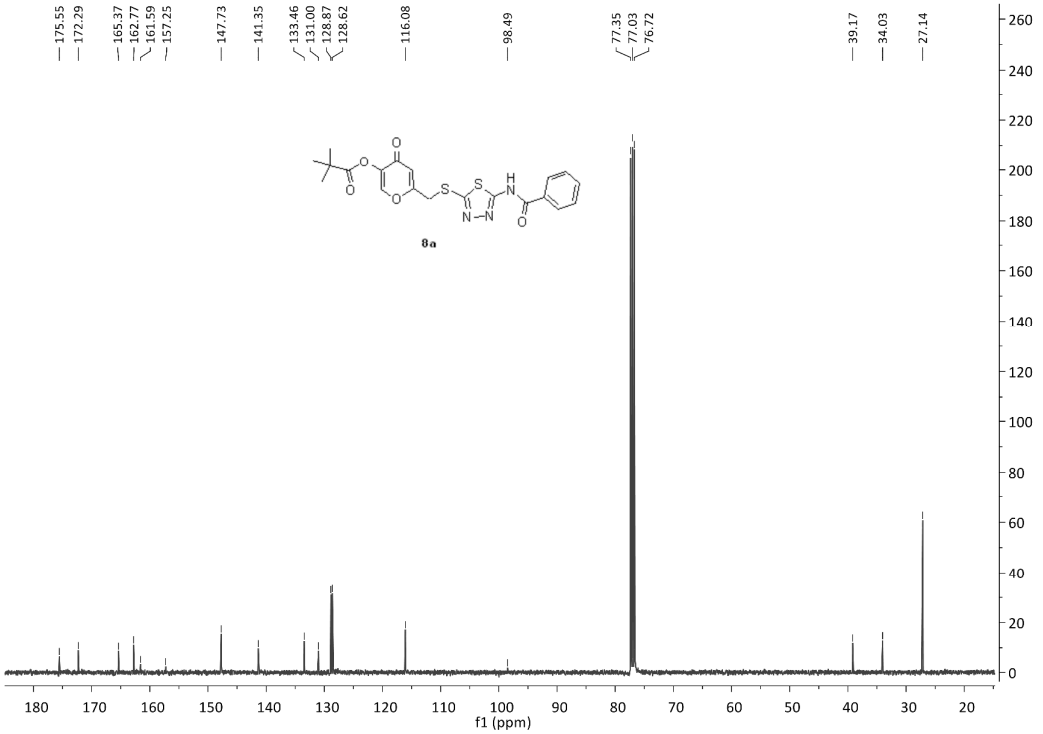


Spectroscopical data for final compounds, 8.

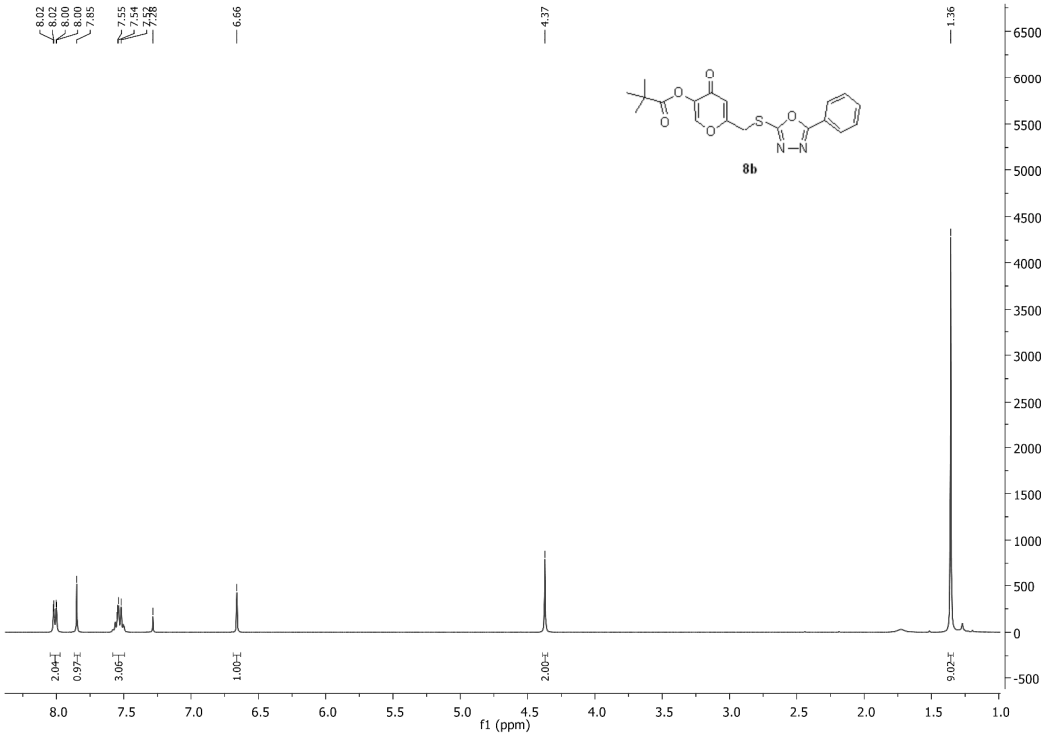
^{1}H-NMR($CDCl_3$) for compound 8a.



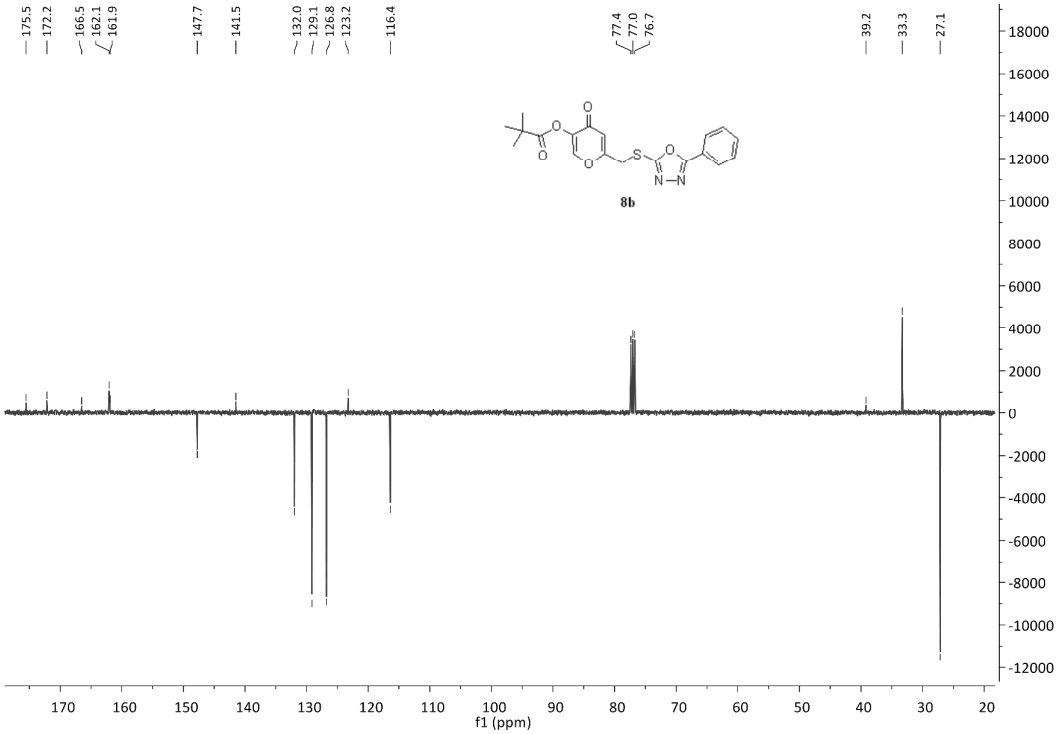
^{13}C-NMR($CDCl_3$) for compound 8a.



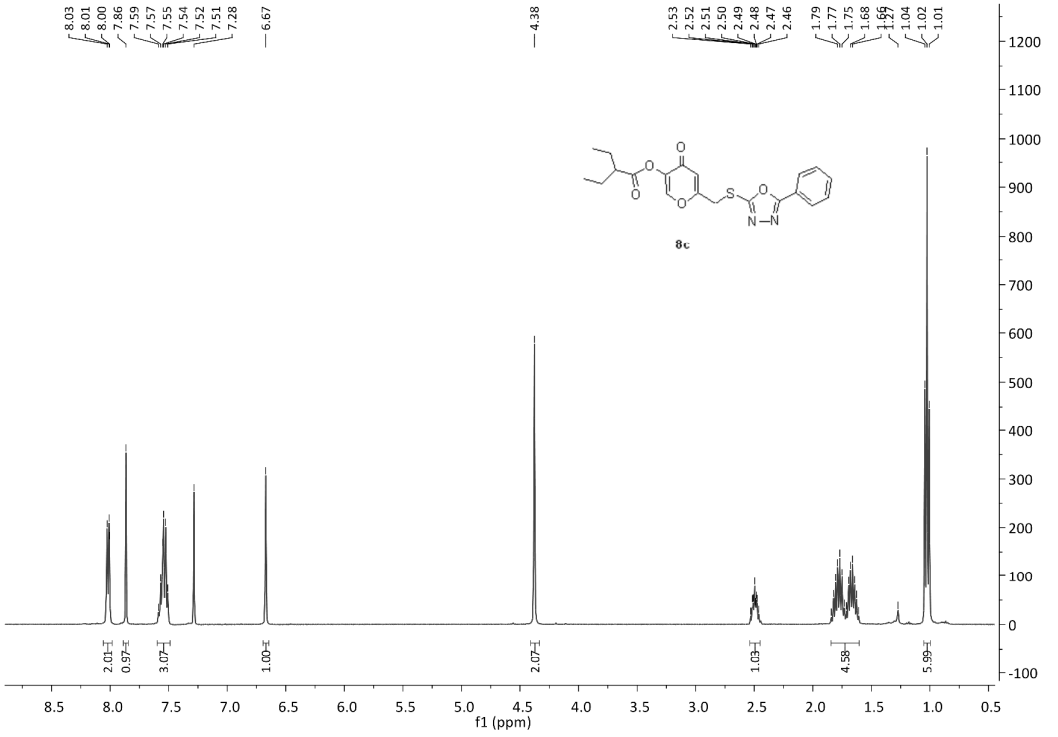
^{1}H-NMR($CDCl_3$) for compound 8b.



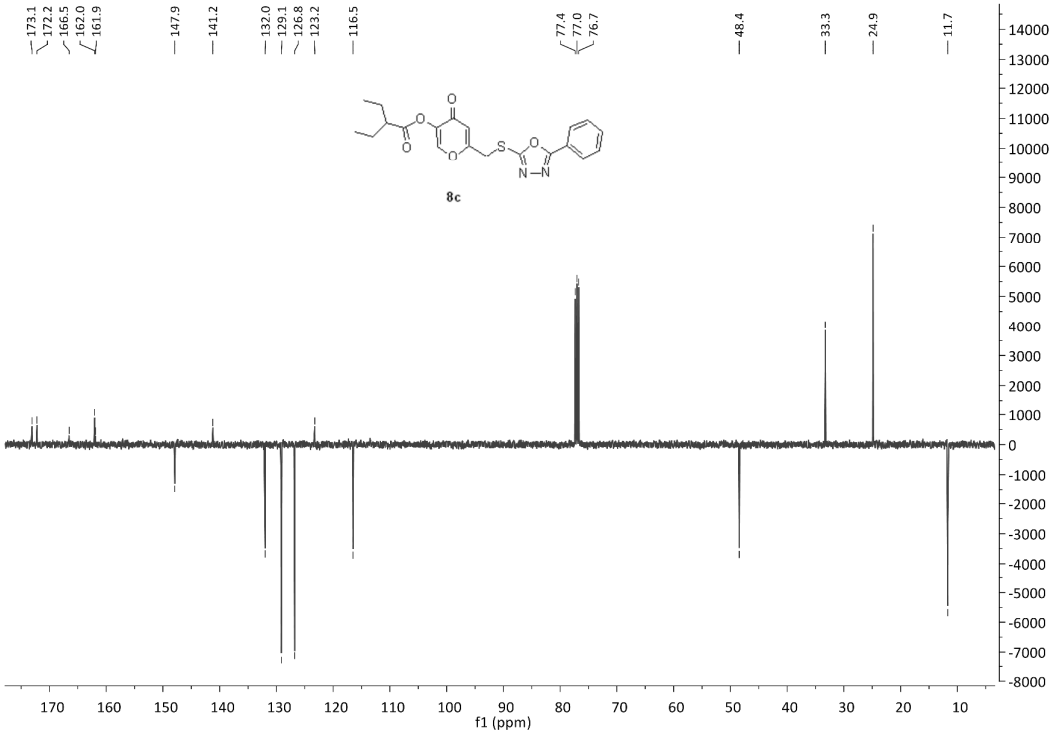
^{13}C-APT-NMR($CDCl_3$) for compound 8b.



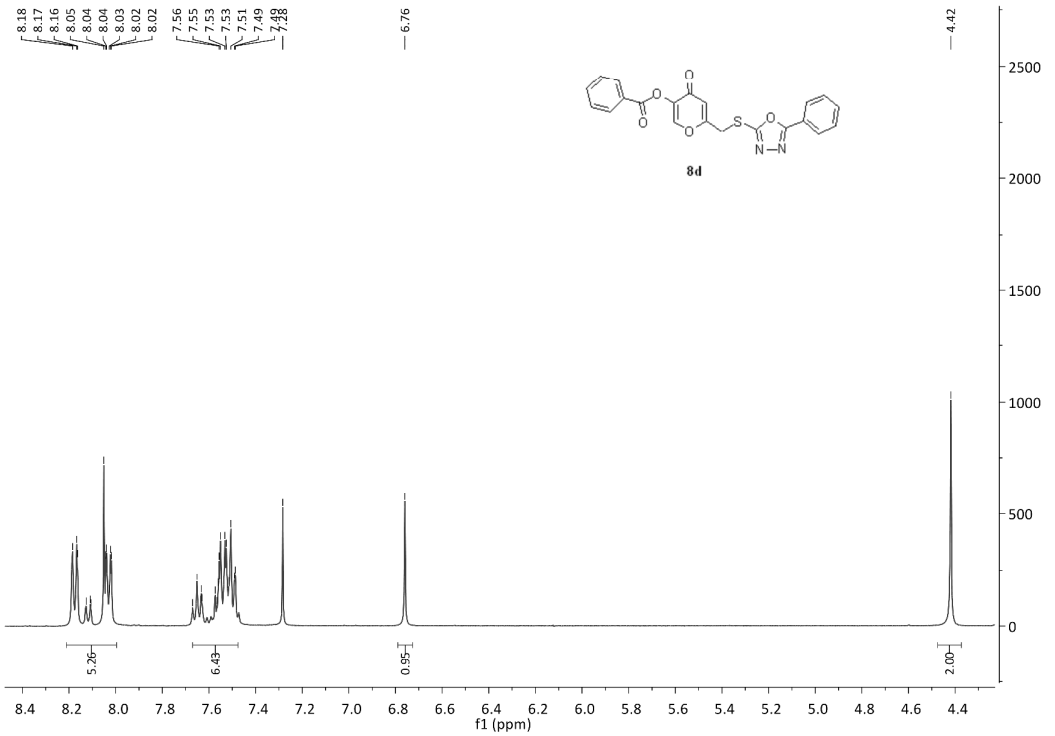
^{1}H-NMR($CDCl_3$) for compound 8c.



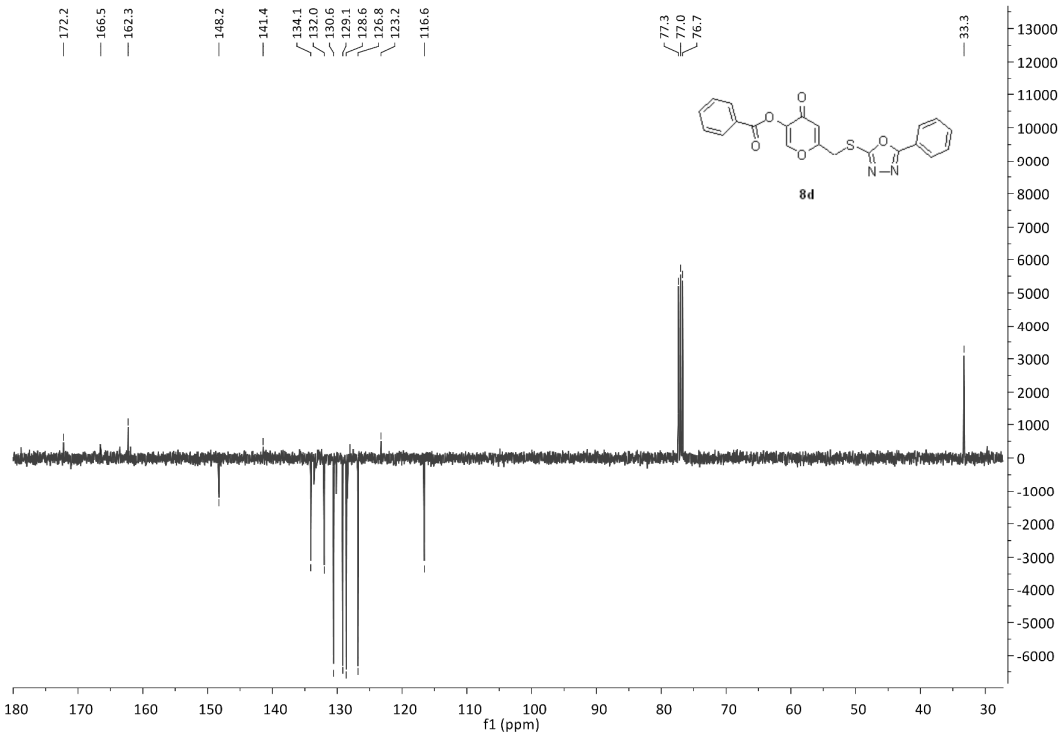
^{13}C-APT-NMR($CDCl_3$) for compound 8c.



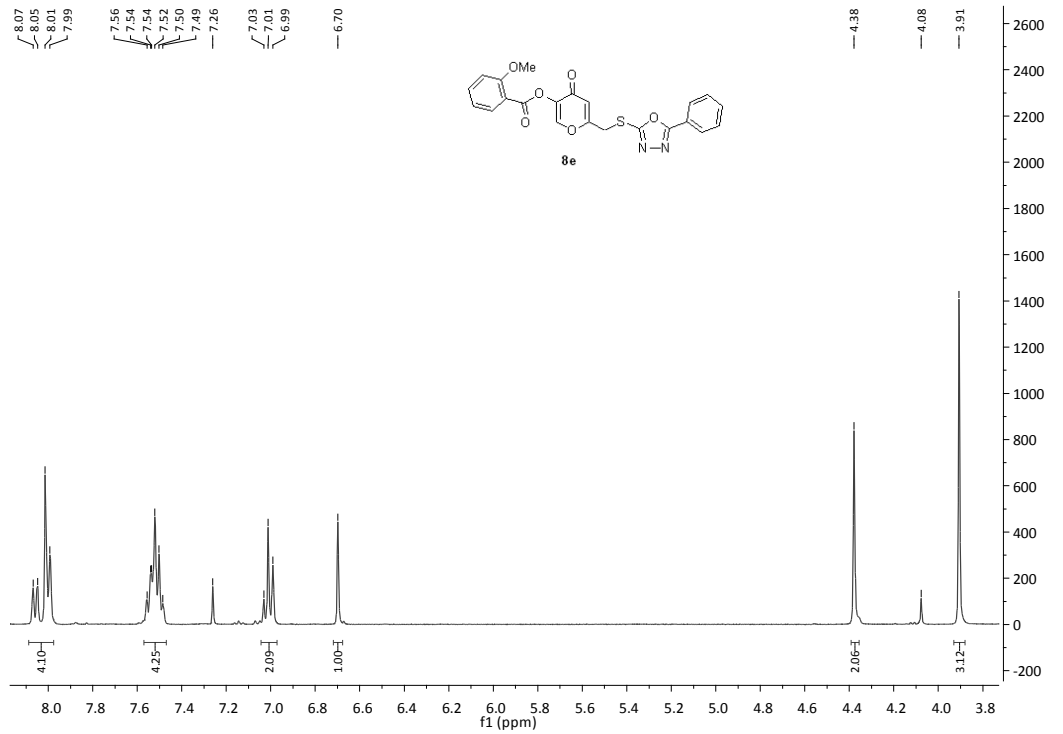
^{1}H-NMR($CDCl_3$) for compound 8d.



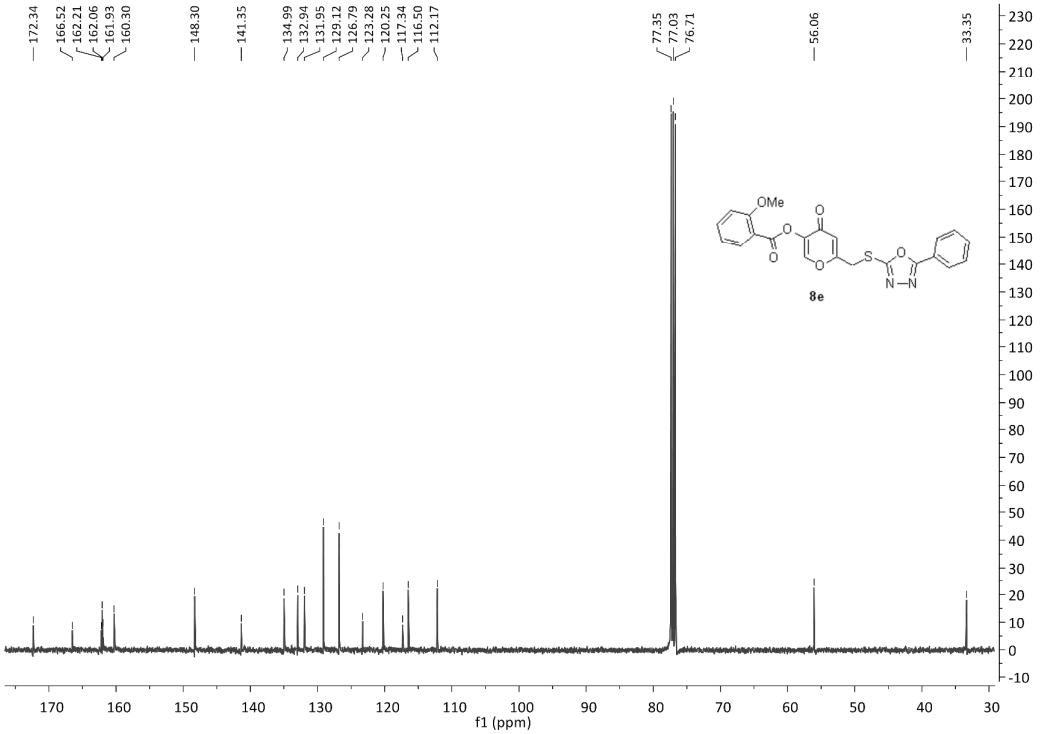
^{13}C-APT-NMR($CDCl_3$) for compound 8d.



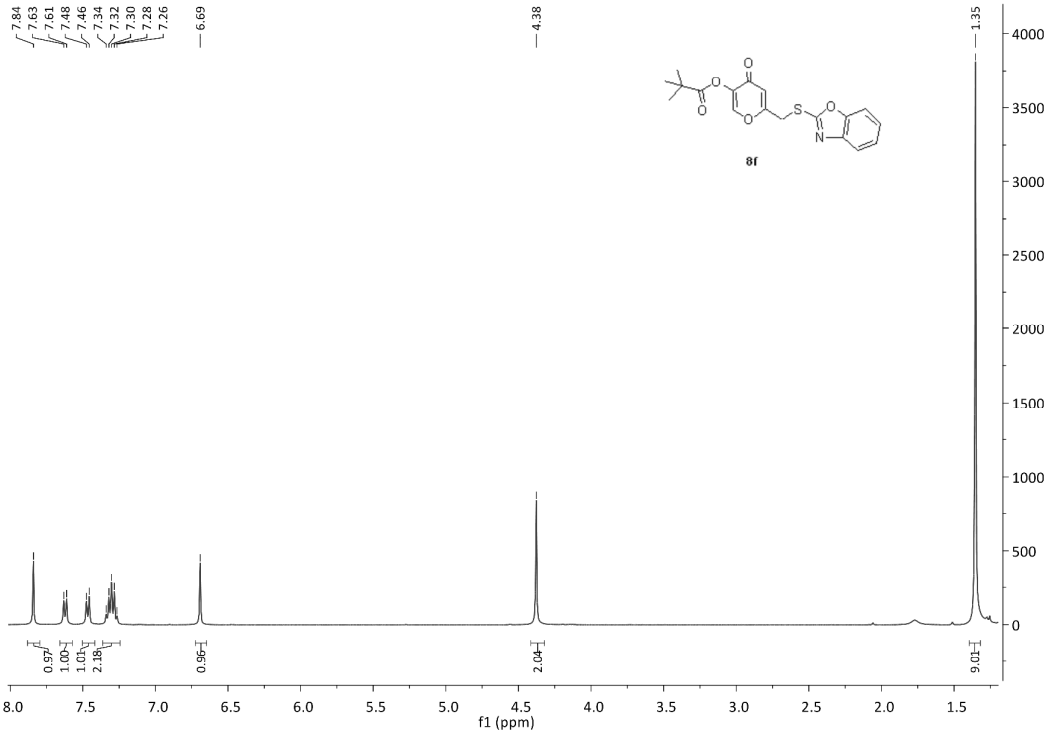
^{1}H-NMR($CDCl_3$) for compound 8e.



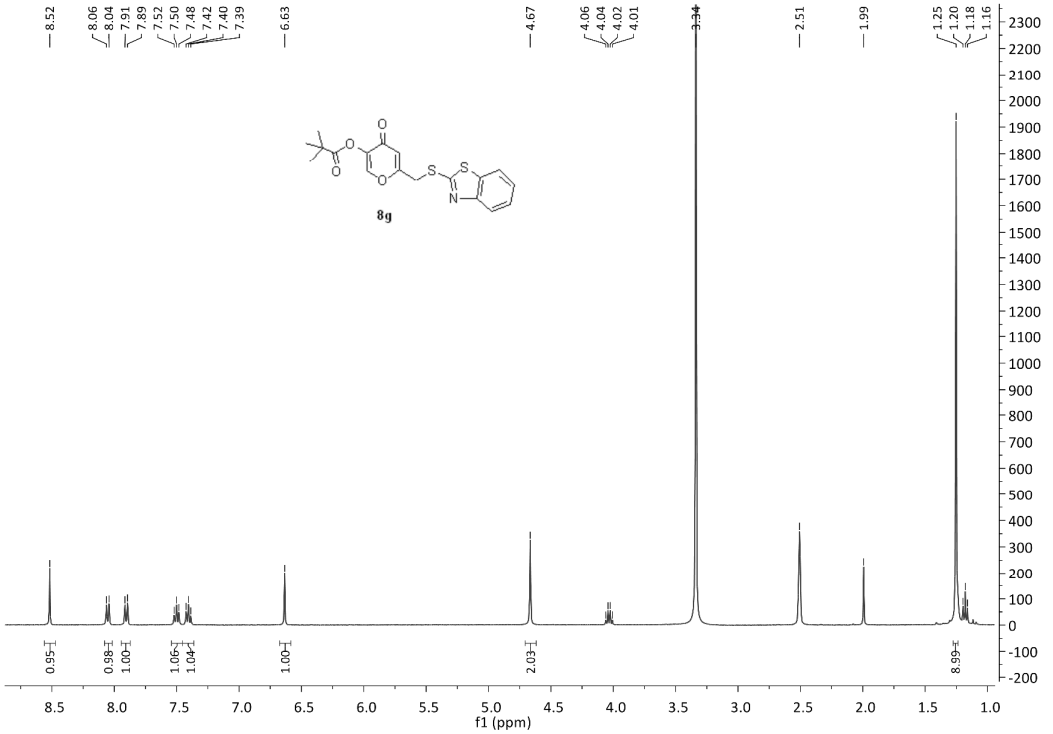
^{13}C-NMR($CDCl_3$) for compound 8e.



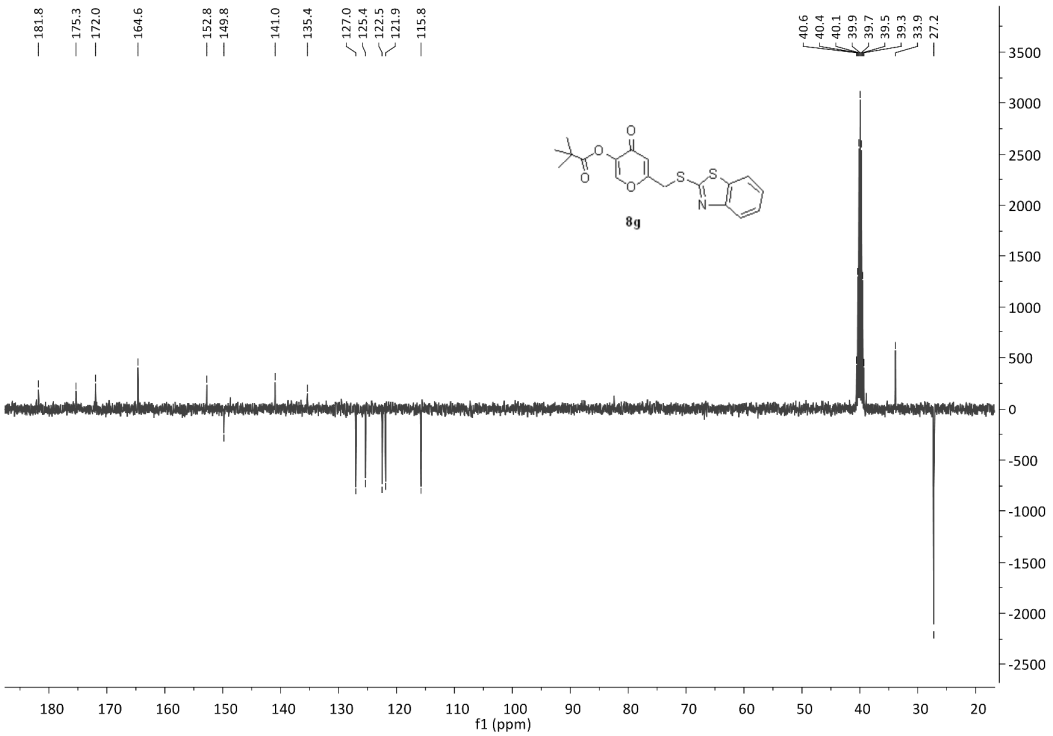
^{1}H-NMR($CDCl_3$) for compound 8f.



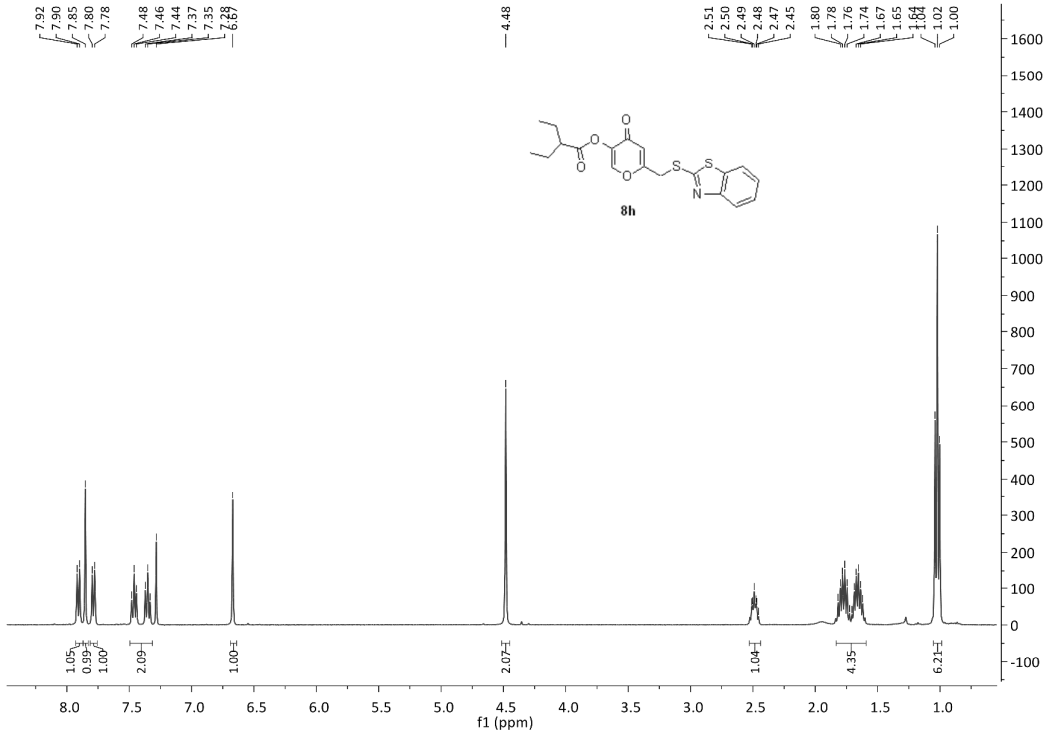
^{1}H-NMR(DMSO) for compound 8g.



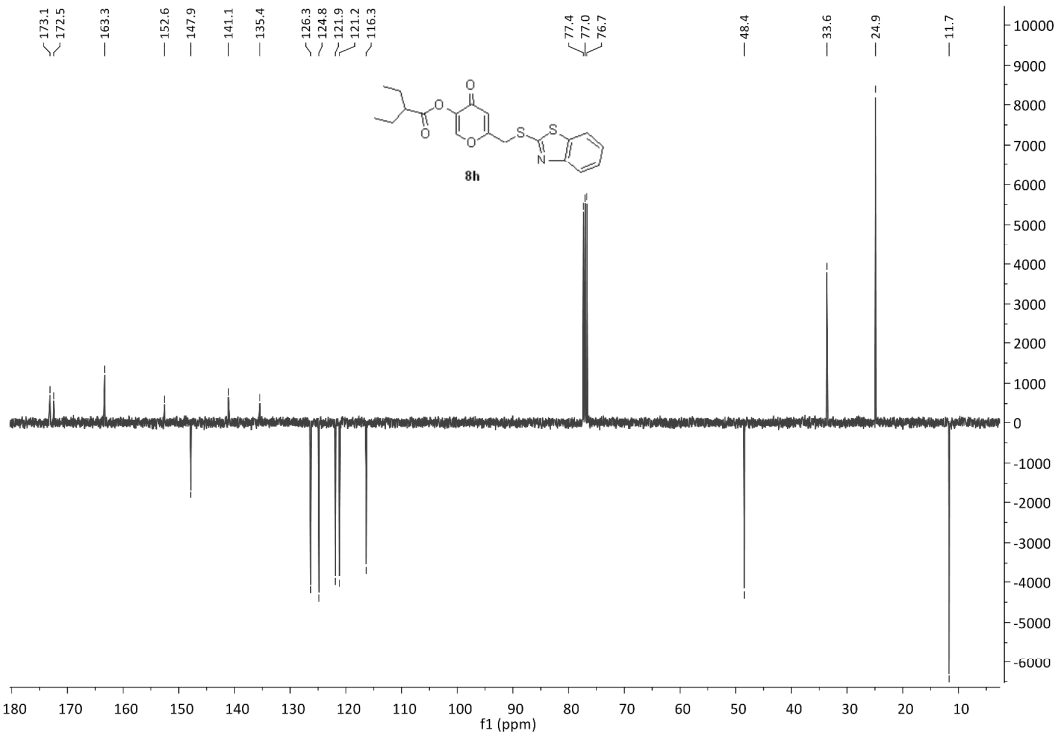
^{13}C-APT-NMR(DMSO) for compound 8g.



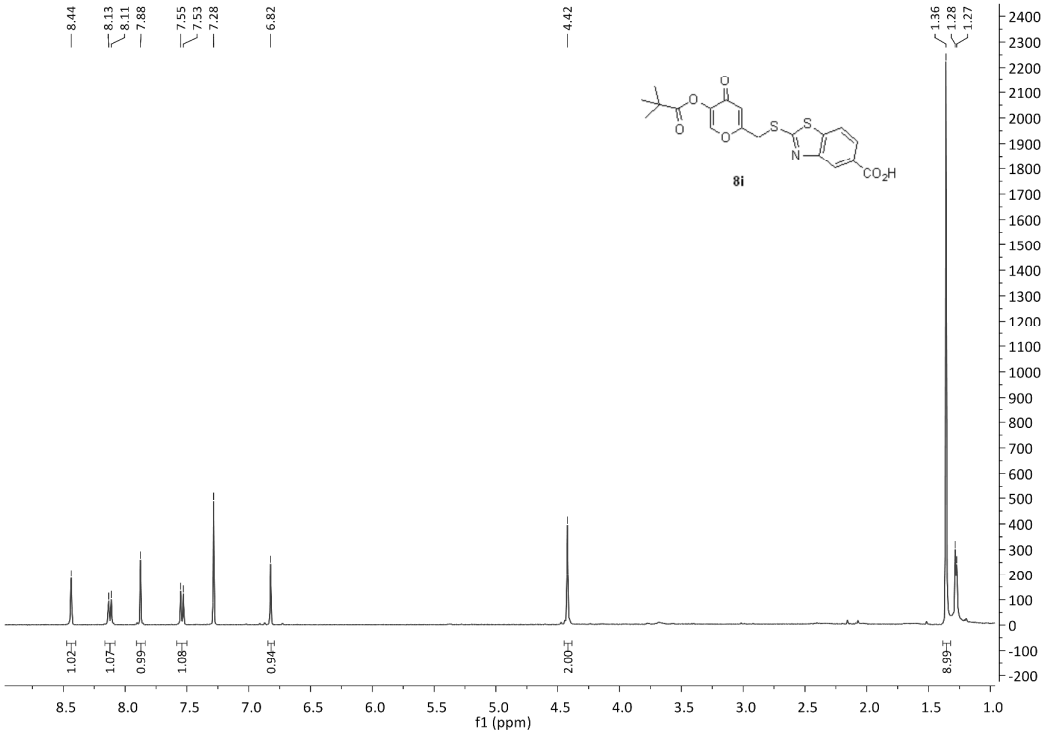
^{1}H-NMR($CDCl_3$) for compound 8h.



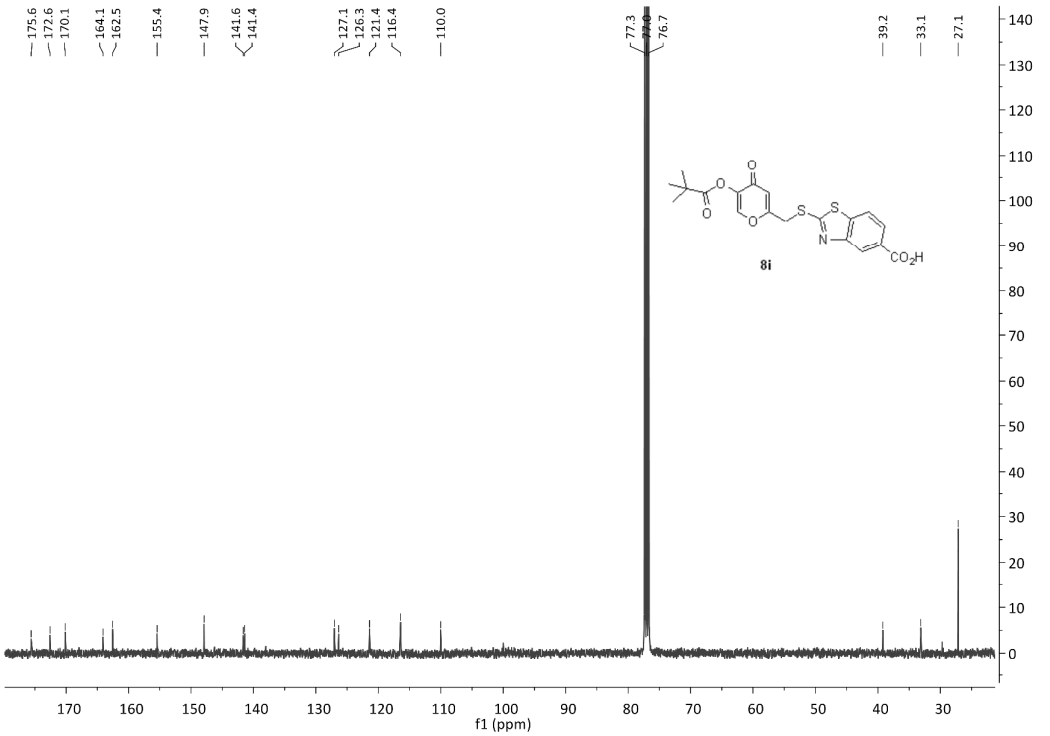
^{13}C-APT-NMR($CDCl_3$) for compound 8h.



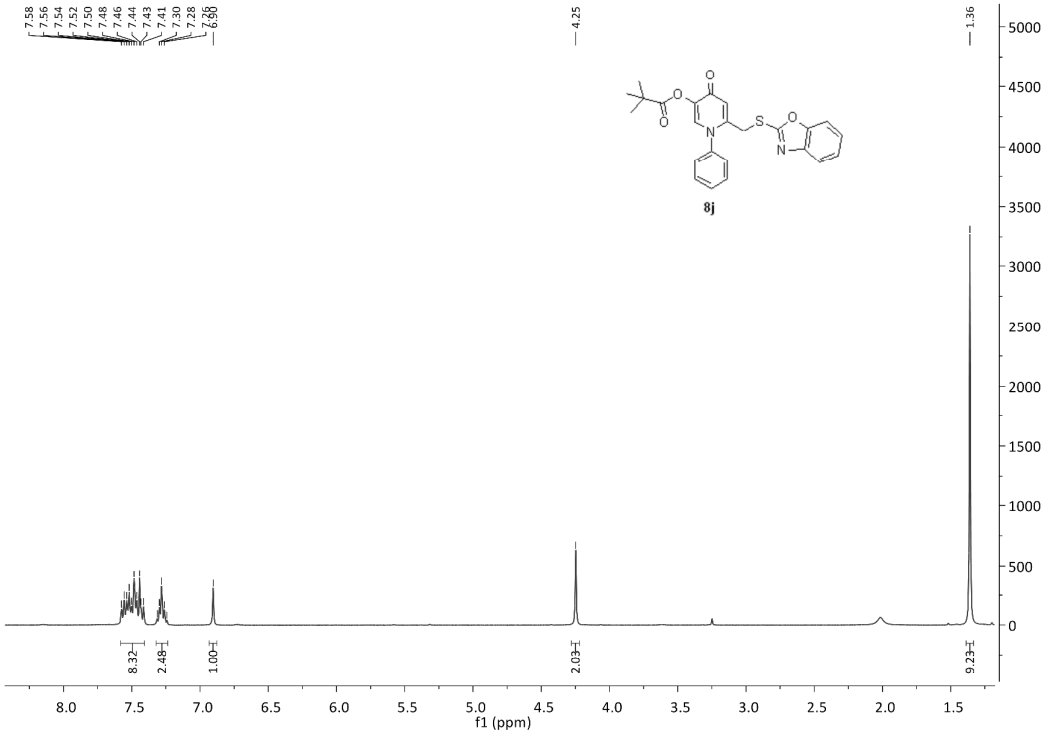
^{1}H-NMR($CDCl_3$) for compound 8i.



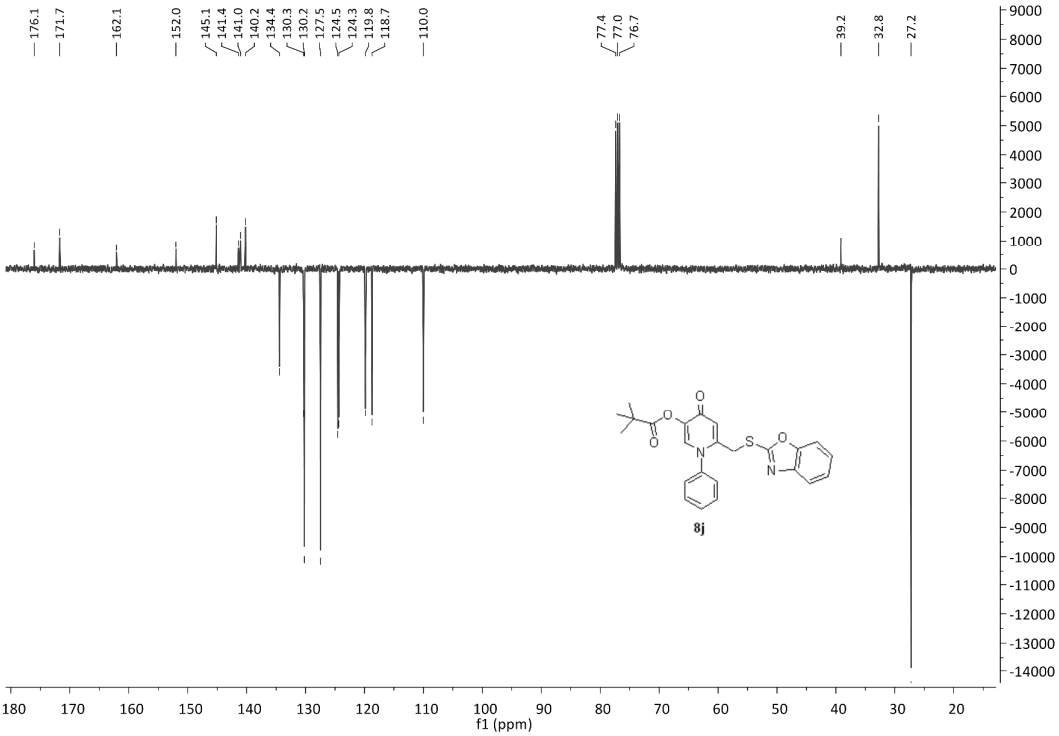
^{13}C -NMR($CDCl_3$) for compound 8i.



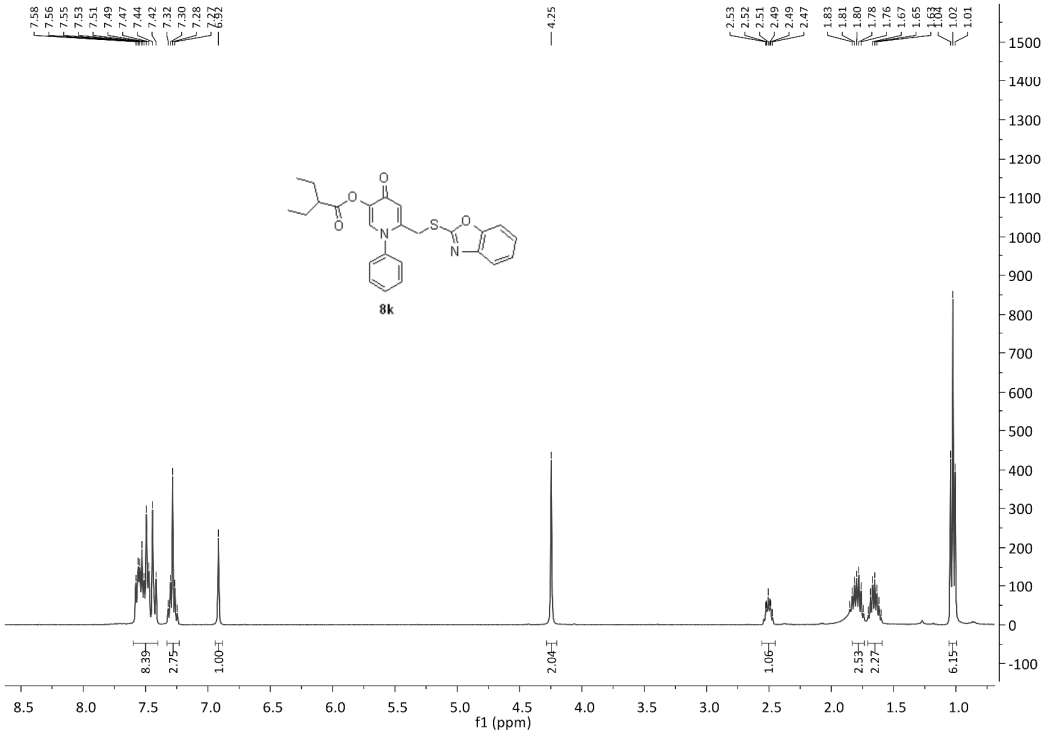
^{1}H-NMR($CDCl_3$) for compound 8j.



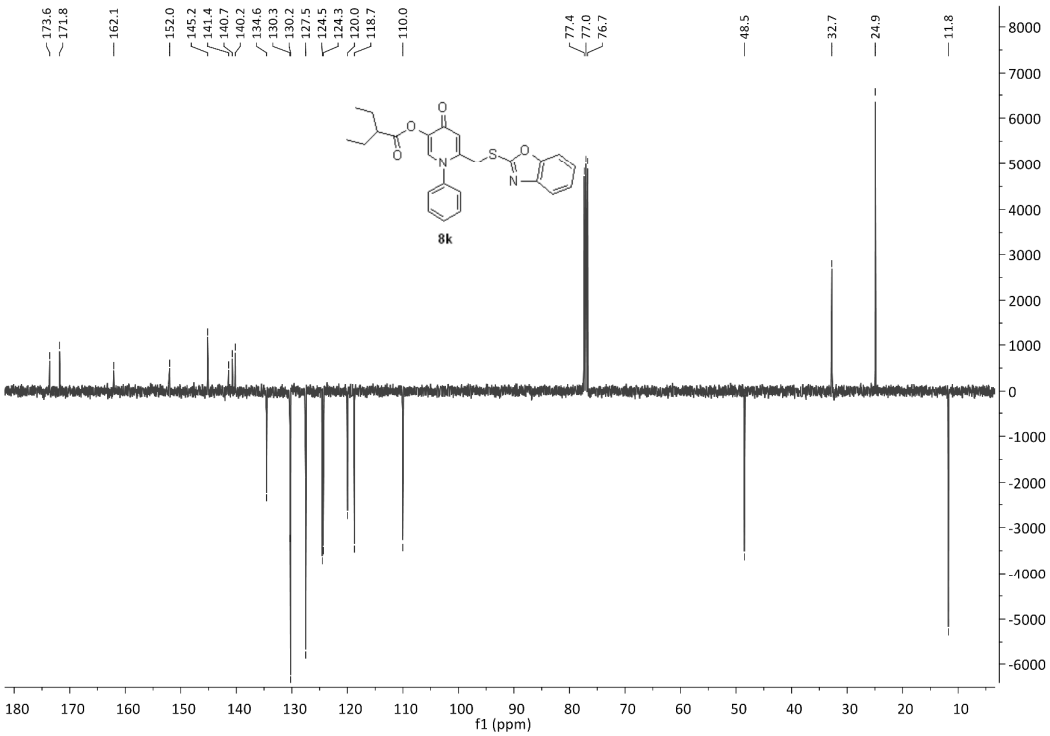
^{13}C-APT -NMR($CDCl_3$) for compound 8j.



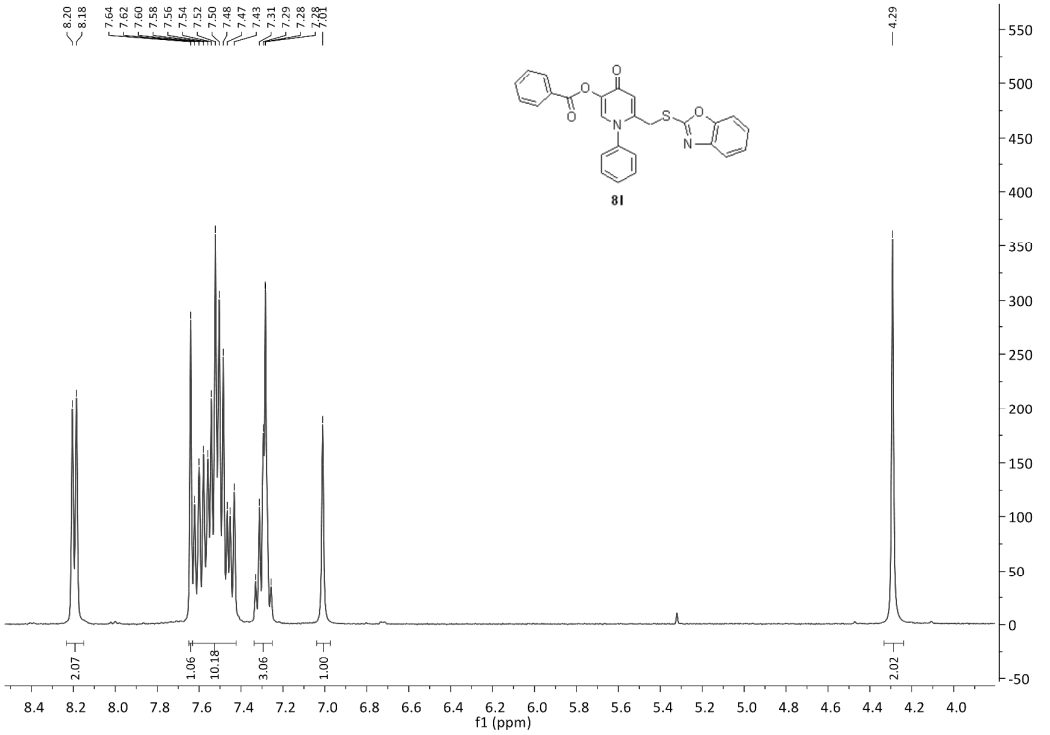
^{1}H-NMR($CDCl_3$) for compound 8k.



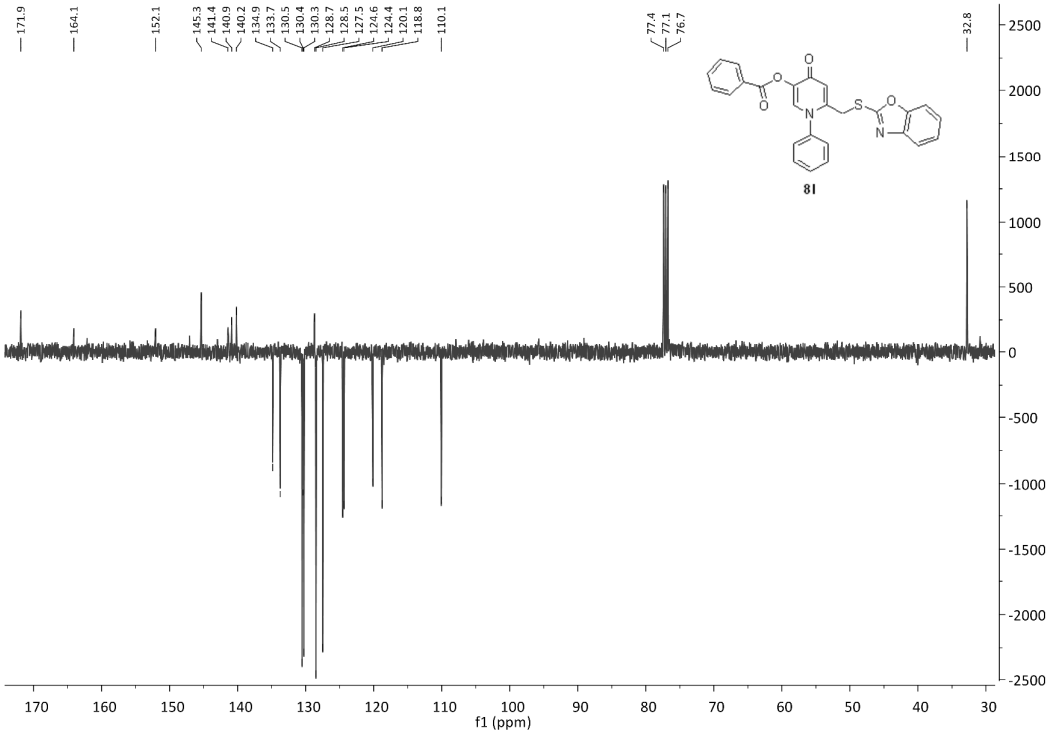
^{13}C-APT -NMR($CDCl_3$) for compound 8k.



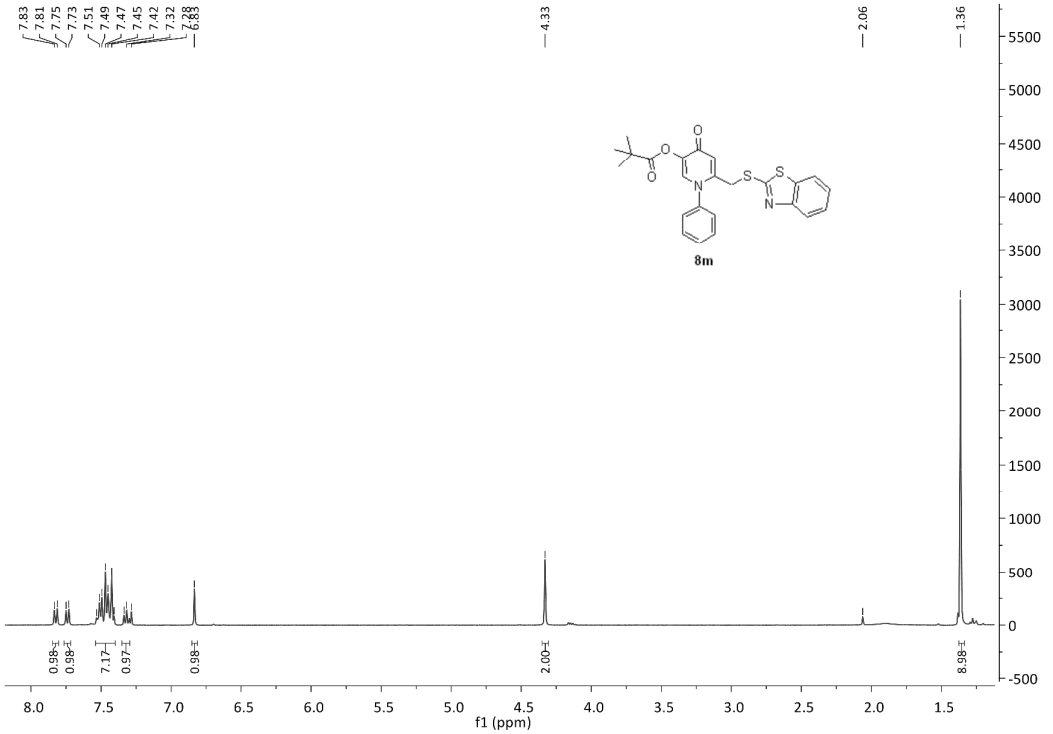
^{1}H-NMR($CDCl_3$) for compound 8l.



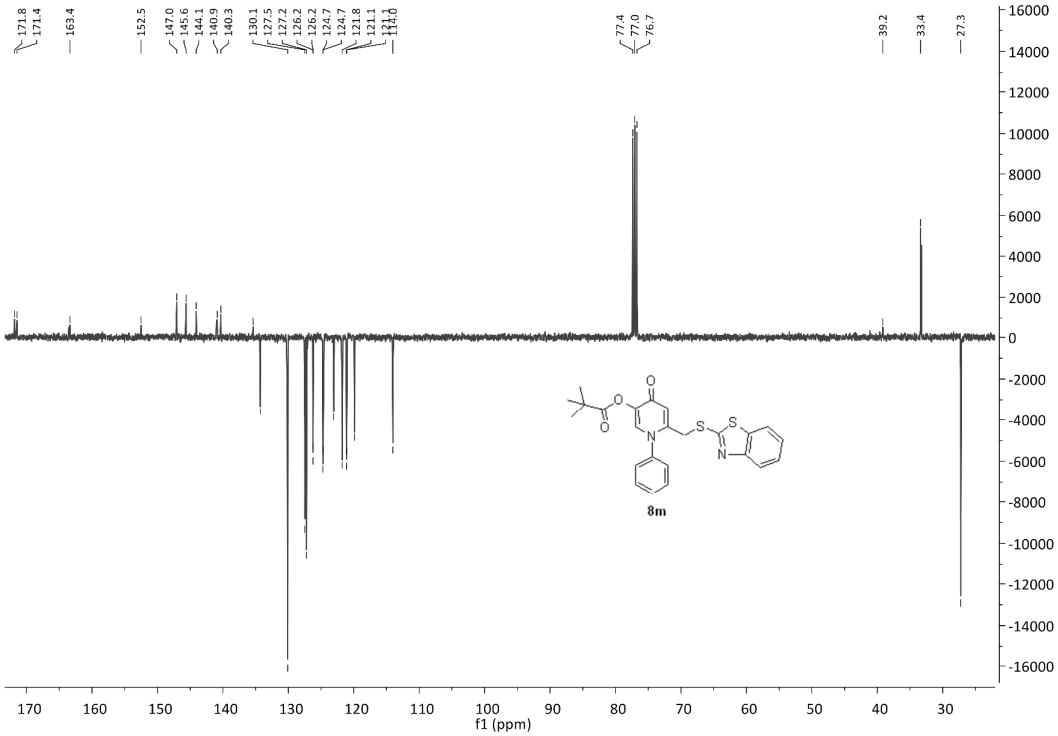
^{13}C-APT -NMR($CDCl_3$) for compound 8l.



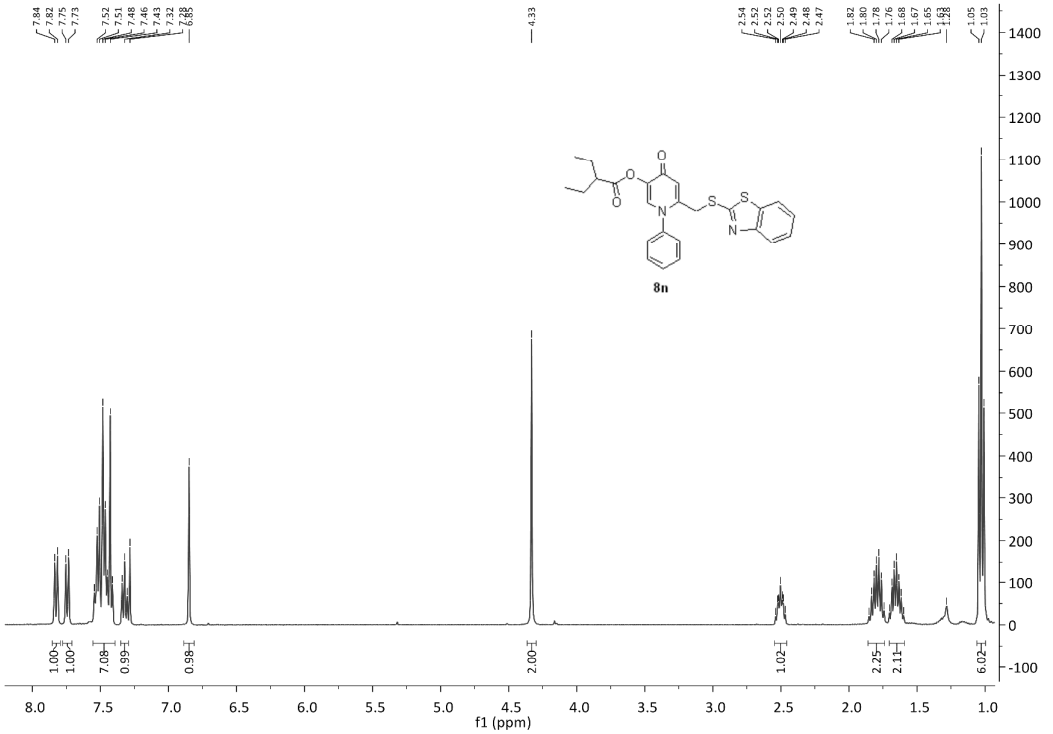
^{1}H-NMR($CDCl_3$) for compound 8m.



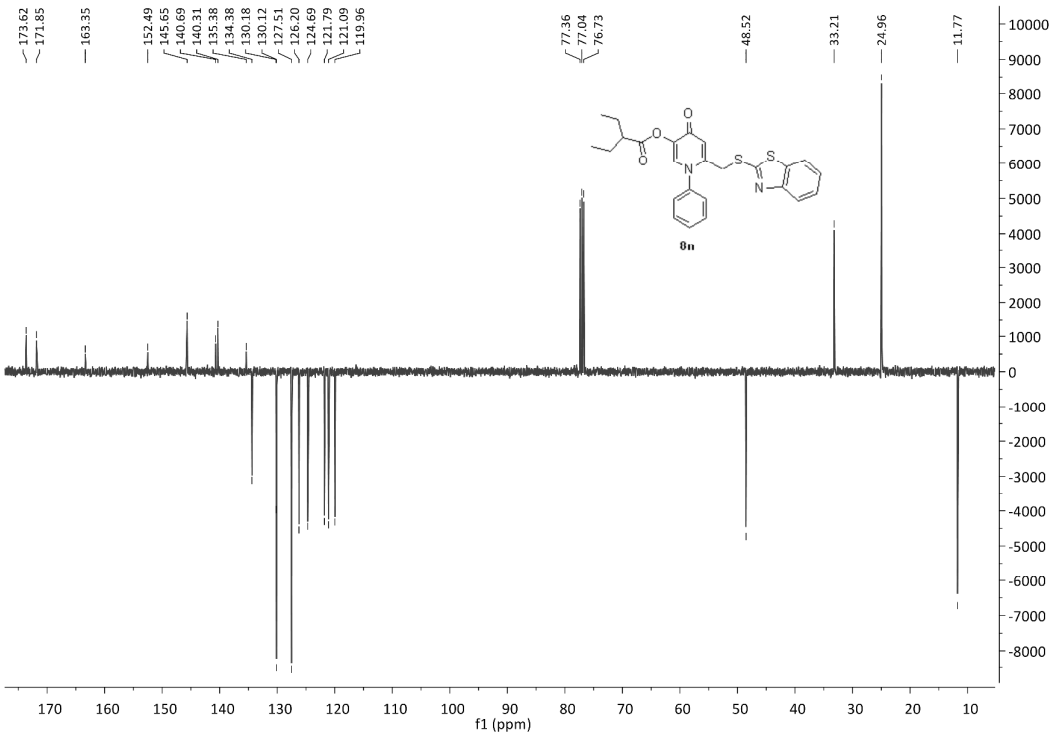
^{13}C-APT-NMR($CDCl_3$) for compound 8m.



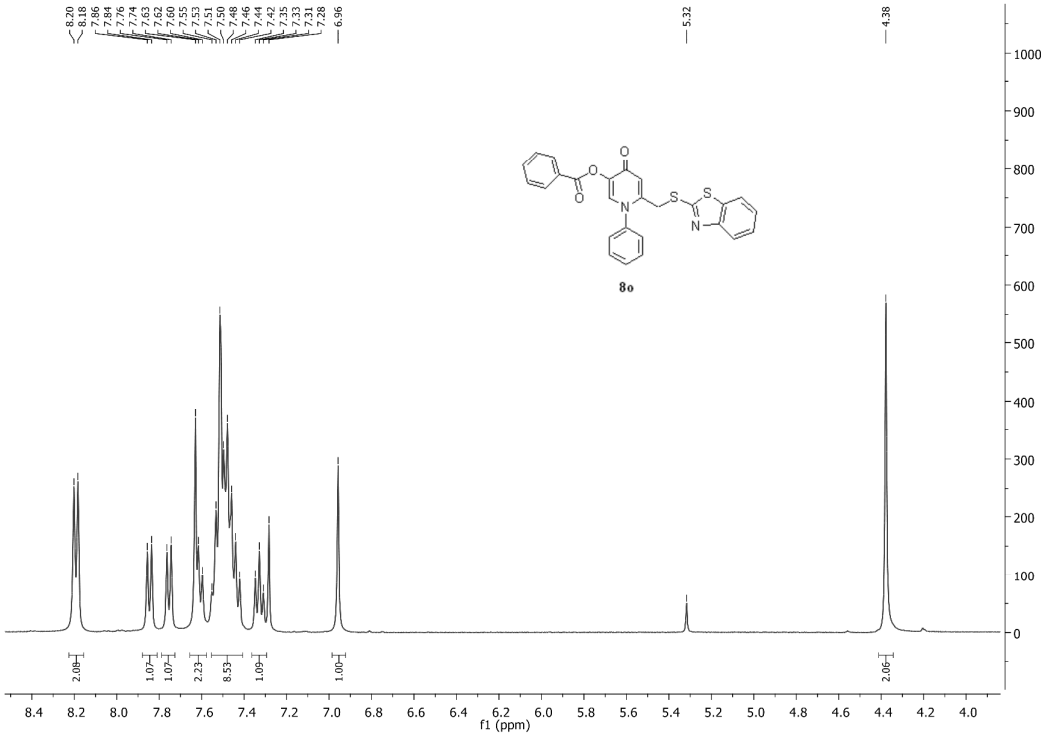
^{1}H-NMR($CDCl_3$) for compound 8n.



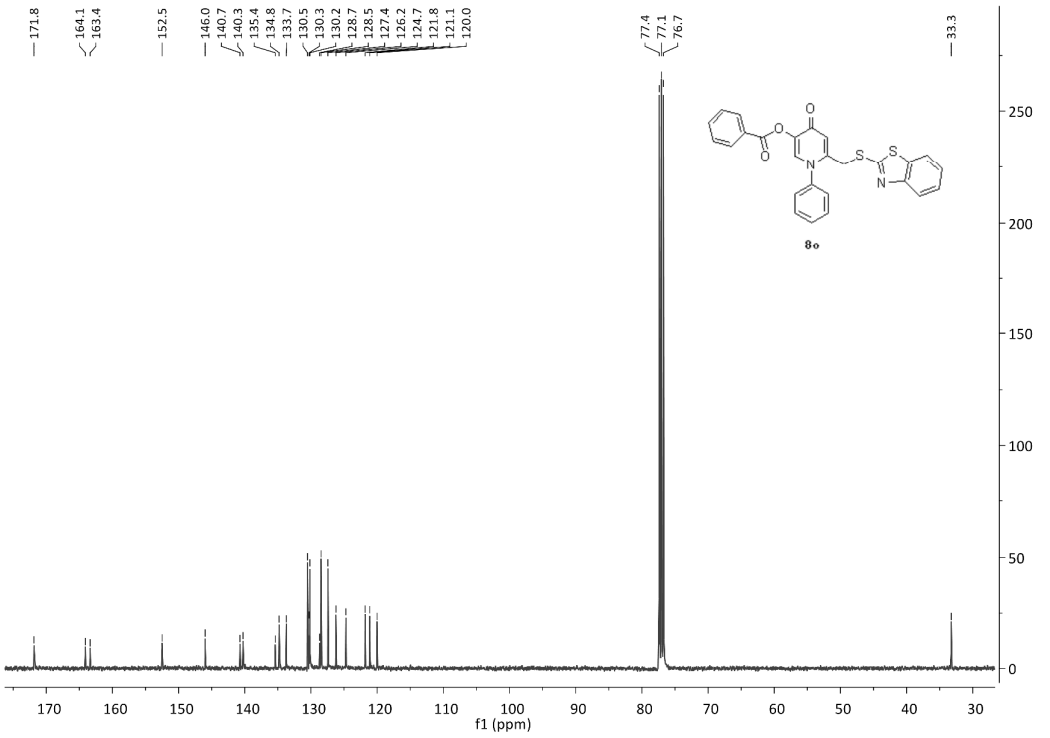
^{13}C-APT-NMR($CDCl_3$) for compound 8n.



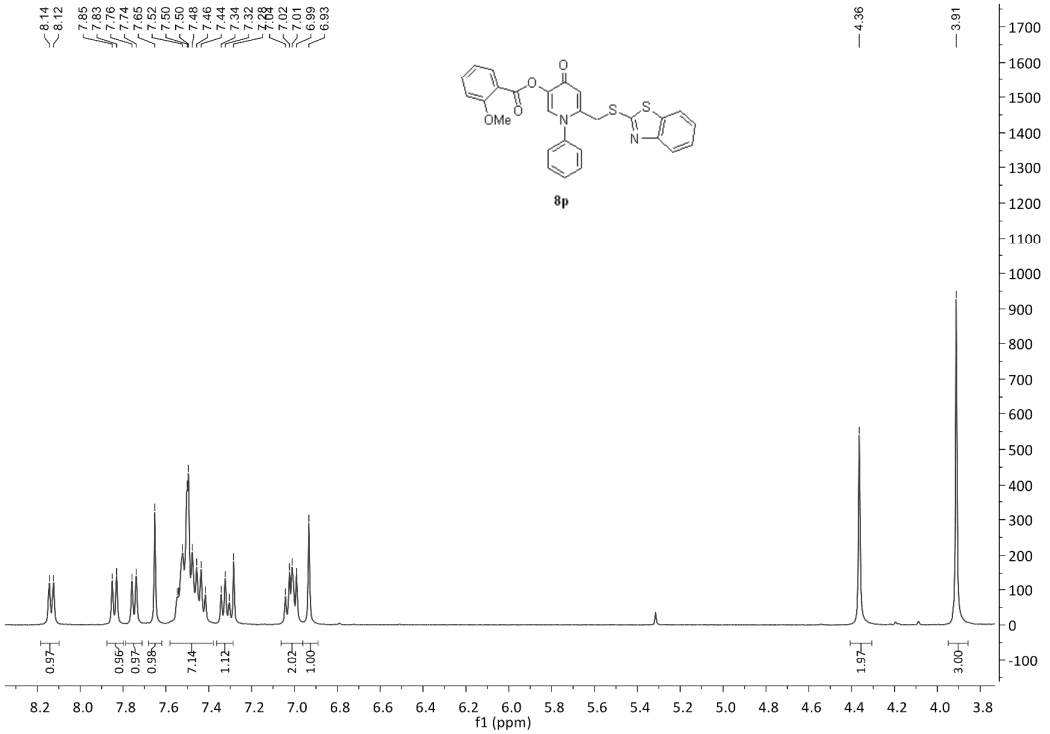
^{1}H-NMR($CDCl_3$) for compound 8o.



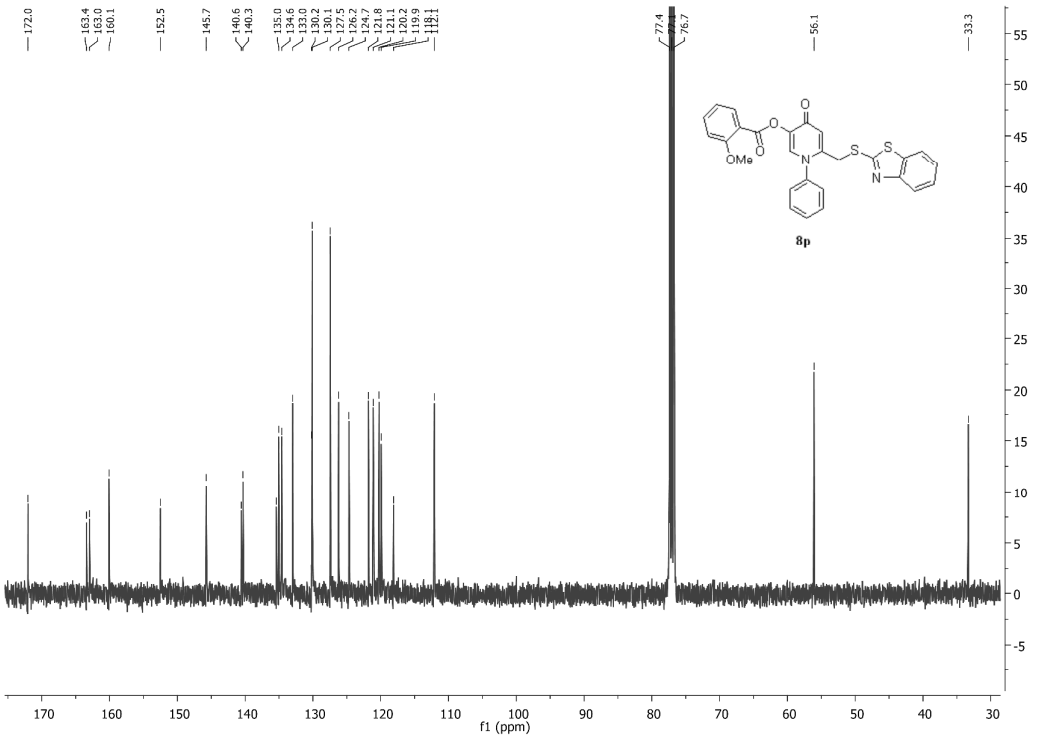
^{13}C-APT-NMR($CDCl_3$) for compound 8o.



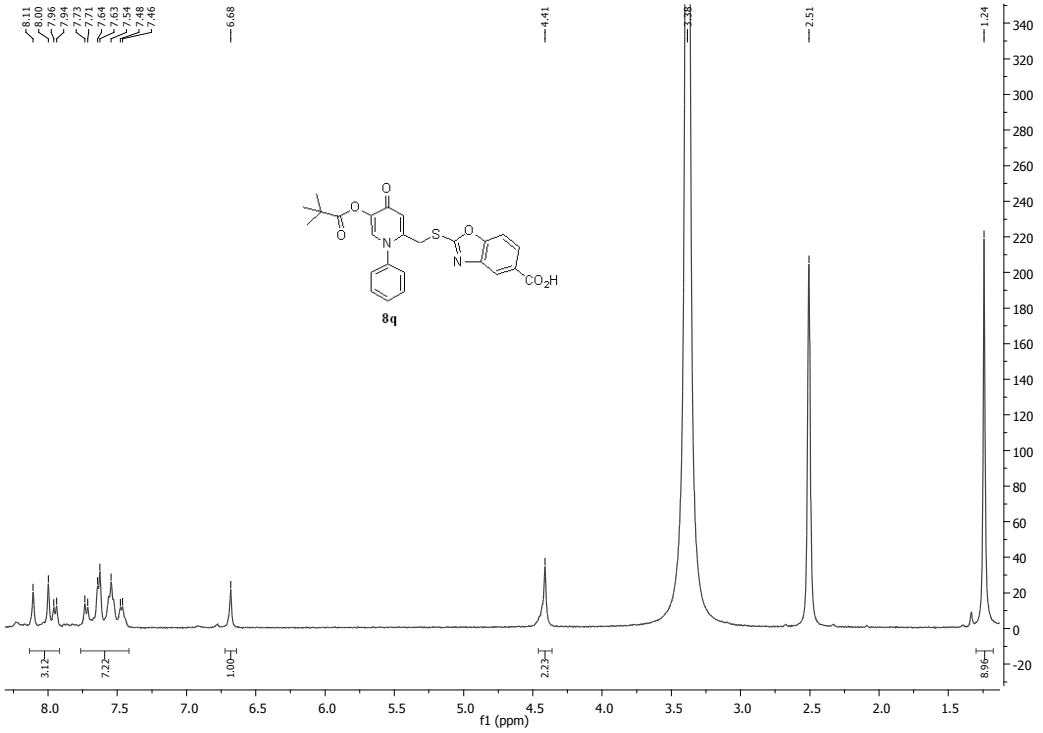
^{1}H-NMR($CDCl_3$) for compound 8p.



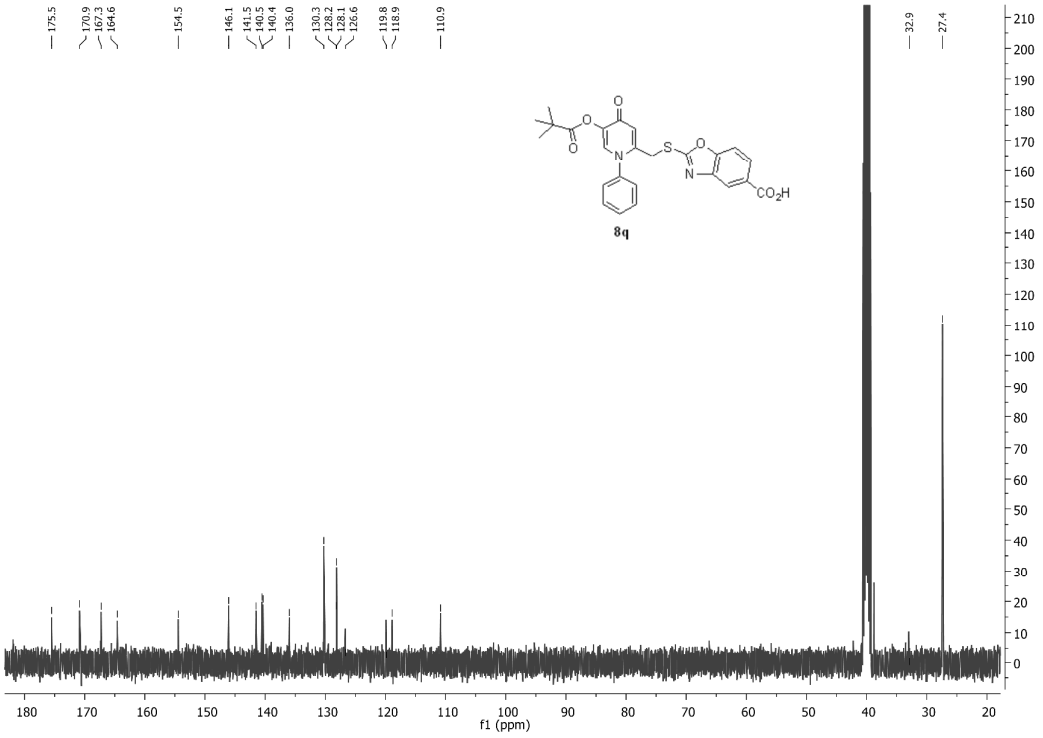
^{13}C-NMR($CDCl_3$) for compound 8p.



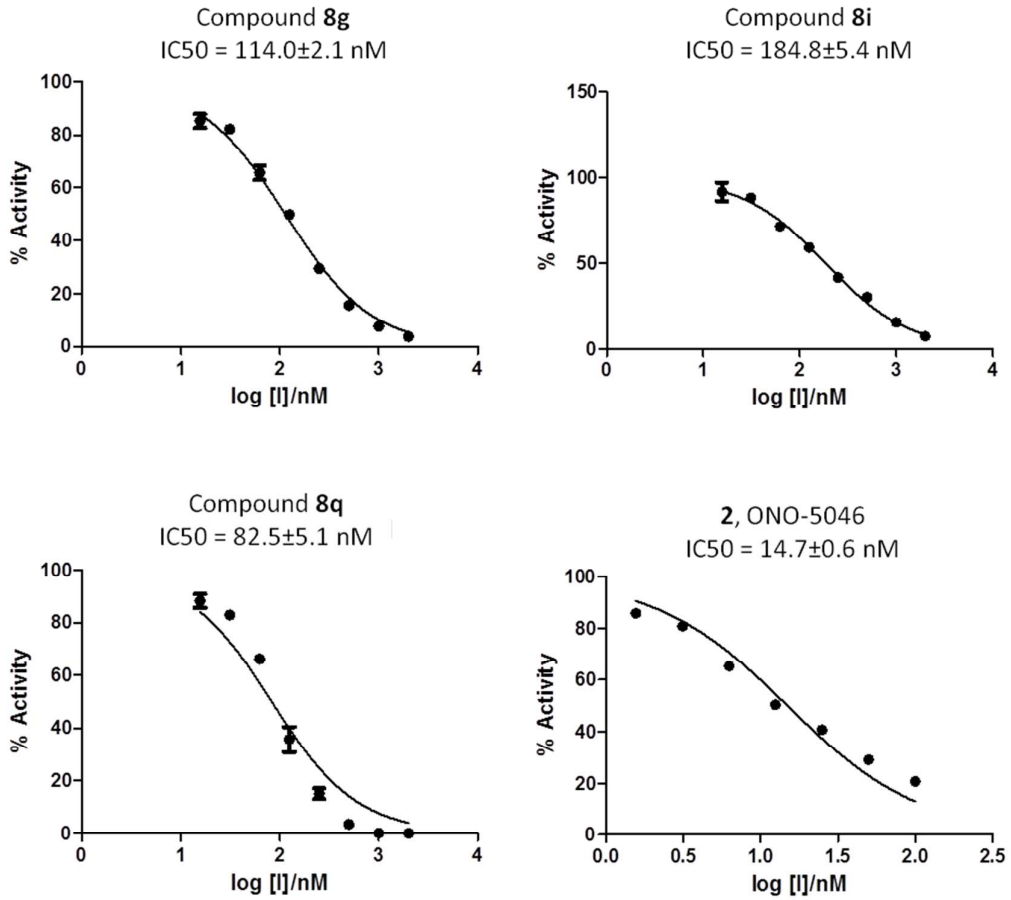
^{1}H-NMR(DMSO) for compound 8q.



^{13}C-NMR(DMSO) for compound 8q.



5.2 Inhibition curves for more active compounds 8g, 8i, 8q and ONO-5046(2).



6. References

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