Electronic Supplementary Material (ESI) for RSC Chemical Biology.

Supplementary Information

A Covalent Compound Selectively Inhibits RNA Demethylase ALKBH5 rather than FTO

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Experimental Procedures

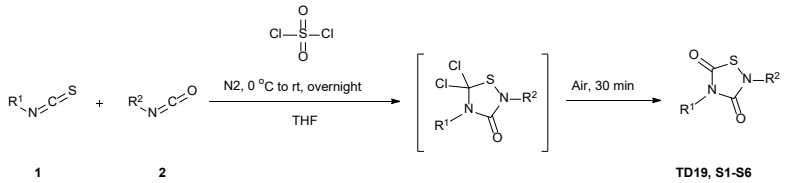
1. Chemistry experiments

General methods

All chemicals were obtained from commercial suppliers and used without further purification unless otherwise noted. The reaction progress was monitored by TLC plates (Yantai Jiangyou Silica gel Development Co., LTD, China) and visualized under UV light (254 nm). NMR spectra were recorded on Bruker ADVANCE III 400 or 500 MHz spectrometers (Germany). Chemical shifts are recorded in ppm relative to tetramethylsilane (TMS, ^{1}H, 0 ppm) or solvent signals: $CDCl_3$ (^{1}H, 7.26 ppm), DMSO-d_6 (^{1}H, 2.50 ppm). NMR peaks are reported as follows: chemical shift, s = singlet, d = doublet, dd = doublet−doublet, t = triplet, q = quartet, m = multiplet, br.s = broad singlet, coupling constant(s) (Hz). Mass spectra (MS) and purity were obtained on an Agilent 1260 Infinity II-IQ LC-MSD system in positive or negative ion mode (ESI). Flow: 1.000 mL/min; Mobile phase (gradient elution): 0-1 min (A/B=90%/10%), 1-5 min (A/B from 90%/10% to 5%/95%), 5-6 min (A/B = 5%/95%), 6-8 min (A/B = 90%/10%), A = water, B = MeCN; Wavelength: 254 nm. Column: InfinityLab Poroshell 120 EC-C18 (2.1 × 50 mm, 2.7 μM).

Synthetic methods of compound TD19 and its derivatives S1-S10

*Preparation of compounds **TD19** and **S1**-**S6***



1 **2** **TD19, S1-S6**

To a stirred mixture of isothiocyanate (**1**, 1.0 equiv), isocyanate (**2**, 1.0 equiv), and anhydrous THF was added sulfuryl chloride (1.0 equiv) at 0 °C. After that, the mixture was warmed to room temperature and stirred under an N_2 atmosphere overnight. Subsequently, the mixture was continued to stir for another 30 min in air. After removing of solvent, the final products **TD19** and **S1**-**S6** could be obtained via silica gel column chromatography with different eluents (eluent: PE: EA = 8: 1 for **TD19**, PE: EA = 9: 1 for **S2**, and PE: EA = 11: 1 for **S5**) directly.

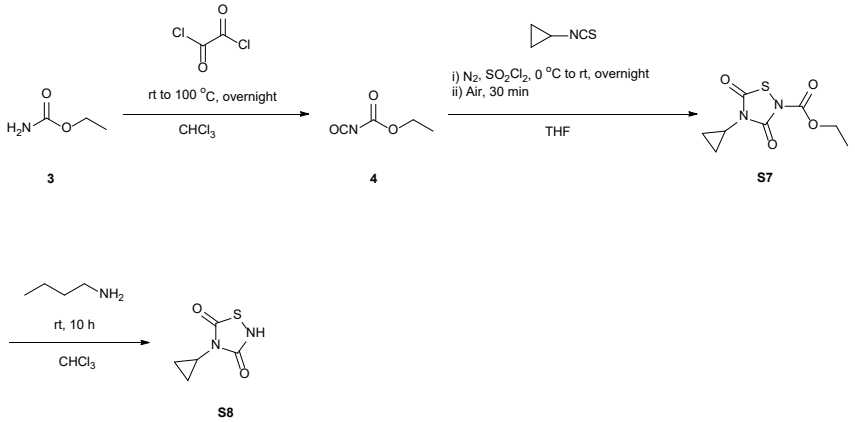
2-Benzyl-4-cyclopropyl-1,2,4-thiadiazolidine-3,5-dione (**TD19**, white solid, yield 62%). ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 7.40-7.27 (5H, m, Ar-H), 4.74 (2H, s, CH_2), 2.76-2.70 (1H, m, CH-cyclopropyl), 1.08-0.97 (4H, m, CH_2-cyclopropyl). ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 166.63, 153.68, 134.63, 129.16 (2C), 128.93, 128.68 (2C), 48.88, 25.22, 5.97 (2C). LC-MS (ESI, *m/z*) calcd for $C_{12}H_{13}N_2O_2S^+$ $[M+H]^+$: 249.07, found 248.9.

2-Benzyl-4-propyl-1,2,4-thiadiazolidine-3,5-dione (**S2**, white solid, yield 68%). ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 7.40-7.30 (5H, m, Ar-H), 4.80 (2H, s, CH_2), 3.56 (2H, t, *J = 7.5 Hz*, -C$\underline{H}_2$CH$_2$CH$_3$), 1.63-1.56 (2H, m, -CH$_2$C$\underline{H}_2$CH$_3$), 0.85 (3H, t, *J = 7.5 Hz*, -CH$_2$CH$_2$C$\underline{H}_3$). ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 165.7, 152.9, 135.5, 128.7 (2C), 128.2, 128.0 (2C), 47.4, 43.6, 20.5, 10.8. LC-MS (ESI, *m/z*) calcd for $C_{12}H_{15}N_2O_2S^+$ $[M+H]^+$: 251.09, found 251.0.

2-Benzyl-4-phenethyl-1,2,4-thiadiazolidine-3,5-dione (**S5**, white solid, yield 55%). ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 7.40-7.26 (5H, m, Ar-H), 7.24-7.16 (5H, m, ArH), 4.76 (2H, s, CH_2), 3.84 (2H, t, *J = 7.5 Hz*, -C$\underline{H}_2$CH$_2$-), 2.92 (2H, t, *J = 7.5 Hz*, -CH$_2$C$\underline{H}_2$-). ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 165.5, 152.6, 137.6, 135.5, 128.7 (2C), 128.7 (2C), 128.4 (2C), 128.1, 127.9 (2C), 126.5, 47.2, 43.1, 32.7. LC-MS (ESI, *m/z*) calcd for $C_{17}H_{17}N_2O_2S^+$ $[M+H]^+$: 313.10, found 313.1.

The characterization data of compounds **S1**, **S3**, **S4**, and **S6** had been reported in our previous study.[1]

*Preparation of compounds **S7** and **S8***

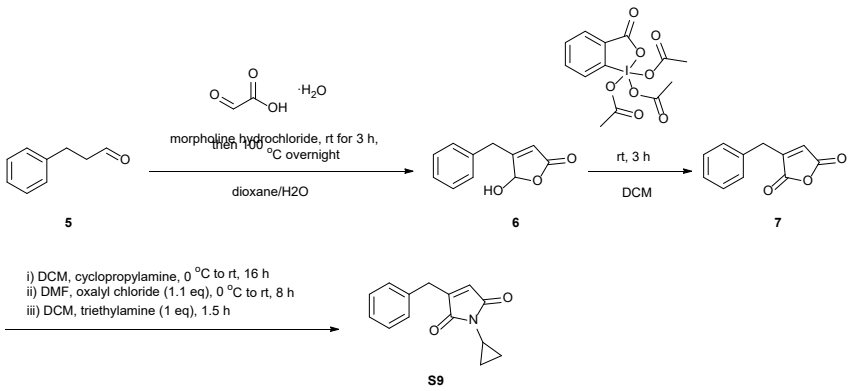


To a solution of ethyl carbamate (**3**, 4.45 g, 49.95 mmol) in $CHCl_3$ (50 mL) was added oxalyl chloride (6.34 mL, 74.92 mmol) at room temperature, and the mixture was allowed to stir till no gas was generated. Then, the reaction temperature was heated to 100 °C and the mixture was stirred overnight. After cooling to room temperature, the mixture was filtered, and the filtrate was concentrated to afford a white viscous liquid (**4**, 4.14 g, yield 72%), which was used in the next step directly without further purification.

The synthetic method of compound **S7** is the same as that of **S1-S6** except that the reaction substrates are different, which using intermediate **4** (8.69 mmol) and cyclopropyl isothiocyanate (8.69 mmol) as starting materials and finally through silica gel column chromatography (eluent: PE: EA = 5: 1) to give a white solid (**S7**, 1.1 g, yield 55%). *ethyl 4-cyclopropyl-3,5-dioxo-1,2,4-thiadiazolidine-2-carboxylate* (**S7**). ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 4.39 (2H, q, *J = 7.0 Hz*, -C$\underline{H_2}$CH$_3$), 2.73-2.69 (1H, m, CH-cyclopropyl), 1.37 (3H, t, *J = 7.0 Hz*, -CH$_2$C$\underline{H_3}$), 1.08-0.97 (4H, m, CH$_2$-cyclopropyl). ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 164.7, 148.7, 148.4, 65.4, 25.1, 14.2, 6.0 (2C). LC-MS (ESI, *m/z*) calcd for $C_8H_{11}N_2O_4^+$ $[M+H]^+$: 231.04, found 231.0.

To a stirred mixture of **S7** (1 g, 4.34 mmol) in $CHCl_3$ (30 mL) was added butylamine (0.5 mL) dropwise. The mixture was then stirred at room temperature for 10 h. After complete consumption of the starting material, the mixture was washed with saturated NaCl (aq.) and the organic layer were collected, and dried with anhydrous $MgSO_4$. Subsequently, the solvents were stripped and the residue was purified via silica gel column chromatography (eluent: DCM: MeOH = 30: 1) to give a yellowish solid (**S8**, 309 mg, yield 45%). *4-cyclopropyl-1,2,4-thiadiazolidine-3,5-dione* (**S8**). ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 9.65 (1H, s, NH), 2.68-2.64 (1H, m, CH-cyclopropyl), 0.91-0.82 (4H, m, CH$_2$-cyclopropyl). ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 168.6, 156.8, 24.0, 5.3 (2C). LC-MS (ESI, *m/z*) calcd for $C_5H_7N_2O_2S^+$ $[M+H]^+$: 159.02, found 158.9.

*Preparation of compound **S9***



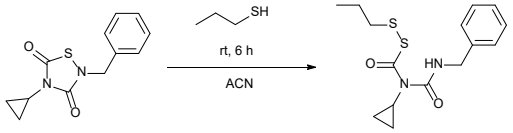
Glyoxylic acid monohydrate (2.3 g, 25 mmol) and morpholine hydrochloride (3.4 g, 27.5 mmol) was dissolved in 25 mL dioxane. 3 mL H_2O was then added dropwise till all the solid material was dissolved. After that, a solution of 3-phenylpropanal (**5**, 3.62 mL, 27.5 mmol) in 5 mL dioxane was added by syringe, and the mixtures were stirred at room temperature for 3 h, followed by heating to 100 °C and stirring overnight till the reaction finished. Then the solvents were removed and extracted with Et_2O three times. The organic layers were combined and washed with aqueous saturated $NaHCO_3$ and brine, dried, and concentrated. Finally, the residue was purified via silica gel column chromatography (eluent: PE: EA = 2: 1) to afford 4-benzyl-5-hydroxyfuran-2(5*H*)-one (**6**, 1.57 g, yield 33%).

To a stirred solution of intermediate **6** (1.0 g, 5.26 mmol) in DCM (20 mL) was added Dess−Martin periodinane (4.46 g, 10.52 mmol), and the mixture was allowed to stir at ambient temperature for 3 h till the starting material disappeared. Subsequently, the solvents

were evaporated and the resulting residues were purified by silica gel column chromatography (eluent: PE: EA = 6: 1) to obtain 3-benzylfuran-2,5-dione (**7**, 247 mg, yield 25%) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 7.14-6.98 (5H, m, Ar-H), 6.17 (1H, s, CH-vinyl), 3.57 (2H, s, CH_2).

Intermediate **7** (100 mg, 0.53 mmol) was suspended in 10 mL DCM, and cyclopropanamine (37 μL, 0.53 mmol) was added dropwise within 5 min at 0 °C. The reaction mixtures were then stirred for 16 h at room temperature and cooled to 0 °C. A few drops of DMF were added and 50 μL of oxalyl chloride (0.58 mmol) was then added in the course of 30 min. The mixtures were continued to stir for another 8 h at room temperature. After that, the excess oxalyl chloride was removed under reduced pressure and the residues were redissolved in 15 mL DCM, and then 74 μL of triethylamine (0.53 mmol) was added. The mixtures were stirred at room temperature for another 1.5 h and filtered. The filtrate was then washed with 1N HCl and the organic layers were combined, dried, concentrated, and purified via silica gel column chromatography (eluent: PE: EA = 7: 1) to give the target product **S9** (45 mg, yield 38%) as a white solid. *3-benzyl-1-cyclopropyl-1H-pyrrole-2,5-dione* (**S9**). ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 7.59 (1H, s, CH-vinyl), 7.49-7.39 (5H, m, Ar-H), 3.53 (2H, s, CH_2), 2.75-2.71 (1H, m, CH-cyclopropyl), 1.04-1.01 (4H, m, CH_2-cyclopropyl). ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 174.6, 171.6, 134.3, 134.2, 130.3 (2C), 130.2, 129.2 (2C), 123.2, 34.1, 22.4, 5.1 (2C). LC-MS (ESI, *m/z*) calcd for $C_{14}H_{14}NO_2^+$ [M+H]$^+$: 228.10, found 228.0.

*Preparation of compound **S10***

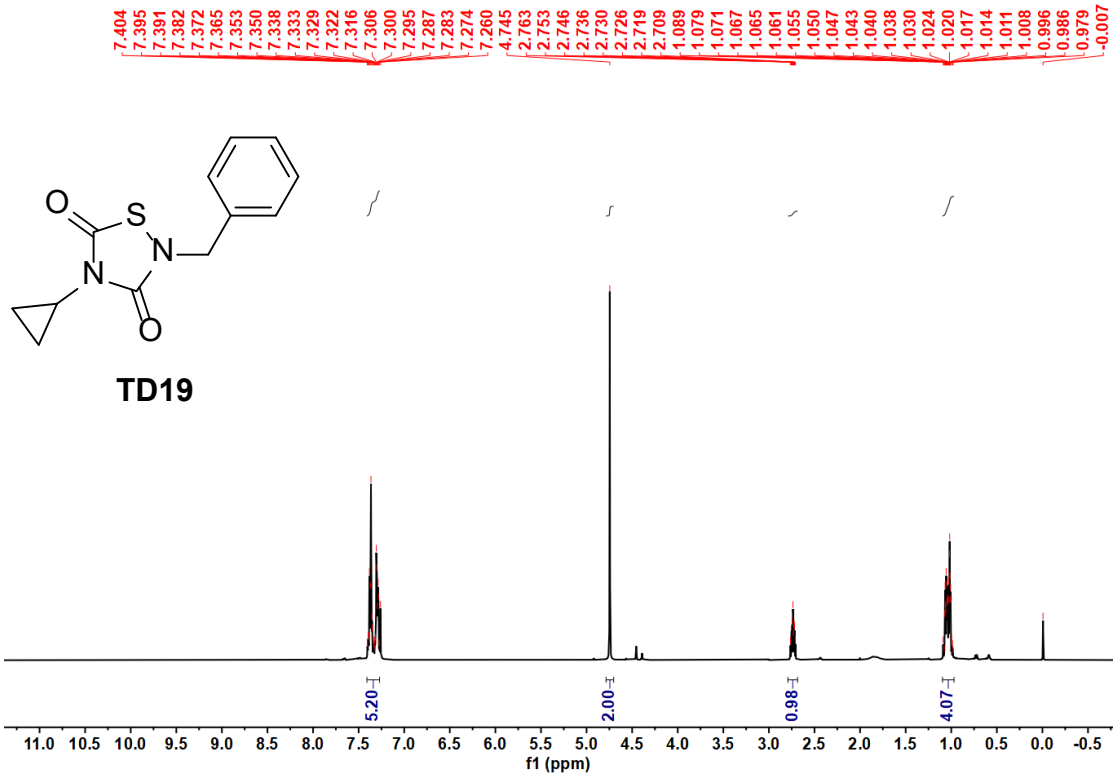


TD19 **S10**

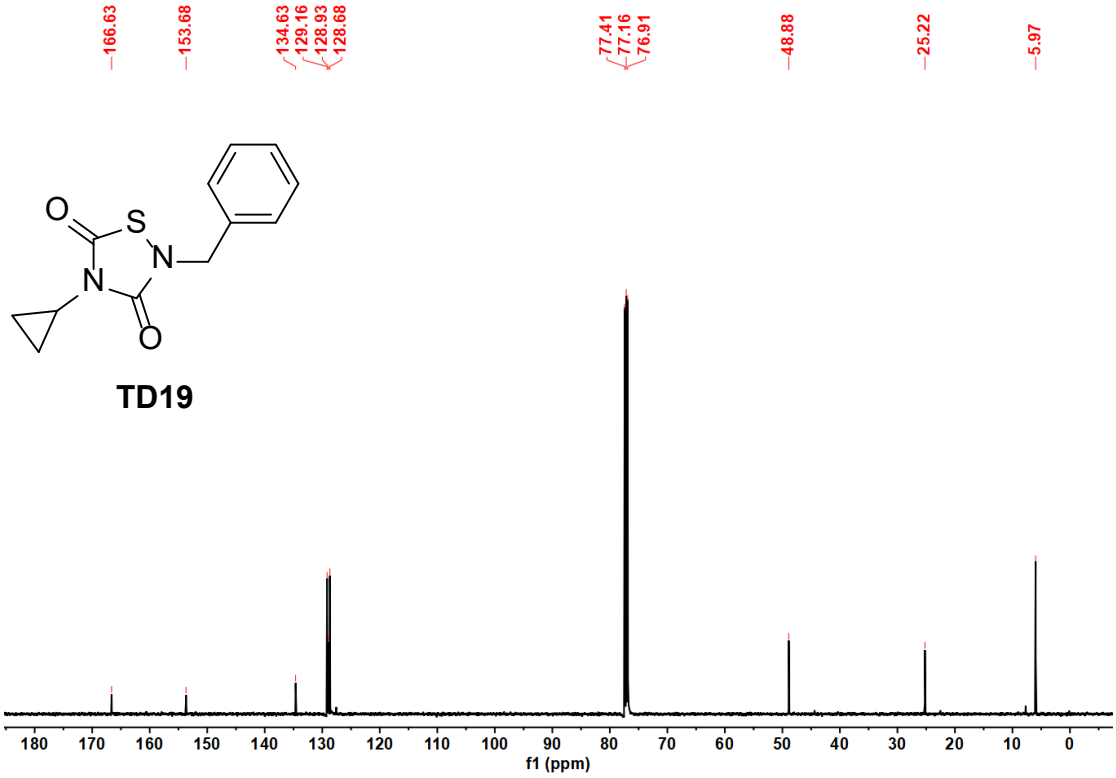
TD19 (100 mg, 0.402 mmol) was dissolved in acetonitrile (10 mL), and then 1-propanethiol (73 μL, 0.805 mmol) was dropwise added to the mixture. After a 6 h stirring for the reaction system at room temperature, solvents were removed and the residue was extracted with EA/H_2O twice. Subsequently, the organic layer was combined, washed with brine, dried, and concentrated. The target product **S10** (47 mg, yield 36%) could be got as a colorless oil using silica gel column chromatography (eluent: PE: EA = 10: 1). *N-[(benzylamino)carbonyl]-N-cyclopropyl-1-(propyldisulfanyl)methanamide* (**S10**). ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 8.44 (1H, t, *J = 6.0 Hz*, NH), 7.35-7.23 (5H, m, Ar-H), 4.34 (2H, d, *J = 6.0 Hz*, CH_2), 2.77-2.72 (1H, m, CH-cyclopropyl), 2.67 (2H, t, *J = 7.0 Hz*, -$C\underline{H}_2CH_2CH_3$), 1.62-1.55 (2H, m, -$CH_2C\underline{H}_2CH_3$), 1.07-1.01 (2H, m, CH_2-cyclopropyl), 0.93 (3H, t, *J = 7.0 Hz*, -$CH_2CH_2C\underline{H}_3$), 0.70-0.67 (2H, m, CH_2-cyclopropyl). ^{13}C NMR (125 MHz, DMSO-d_6) δ (ppm): 169.1, 154.3, 138.8, 128.3 (2C), 127.1 (2C), 126.9, 43.6, 39.6, 27.0, 21.6, 12.7, 10.3 (2C). LC-MS (ESI, *m/z*) calcd for $C_{15}H_{21}N_2O_2S_2^+$ [M+H]$^+$: 325.10, found 325.1.

NMR and LC-MS spectra

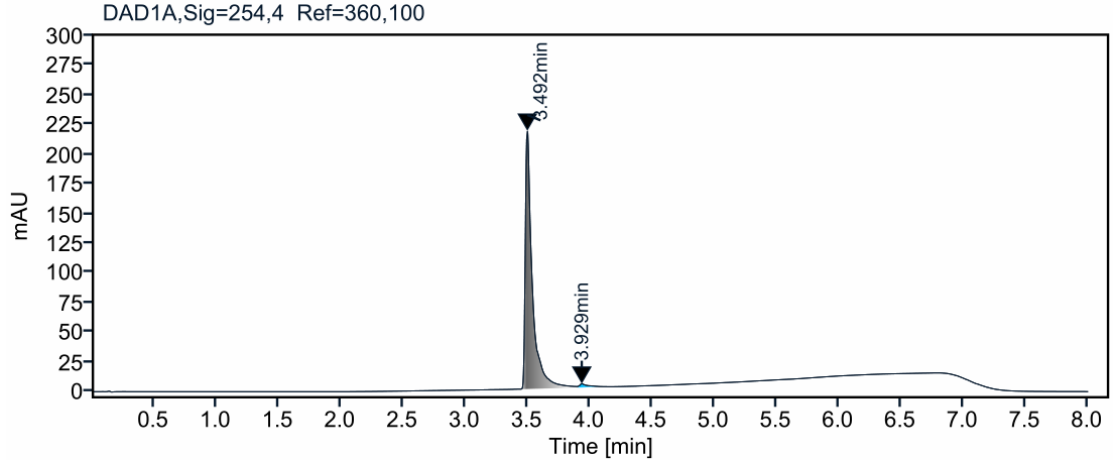
^{1}H NMR spectra of **TD19**

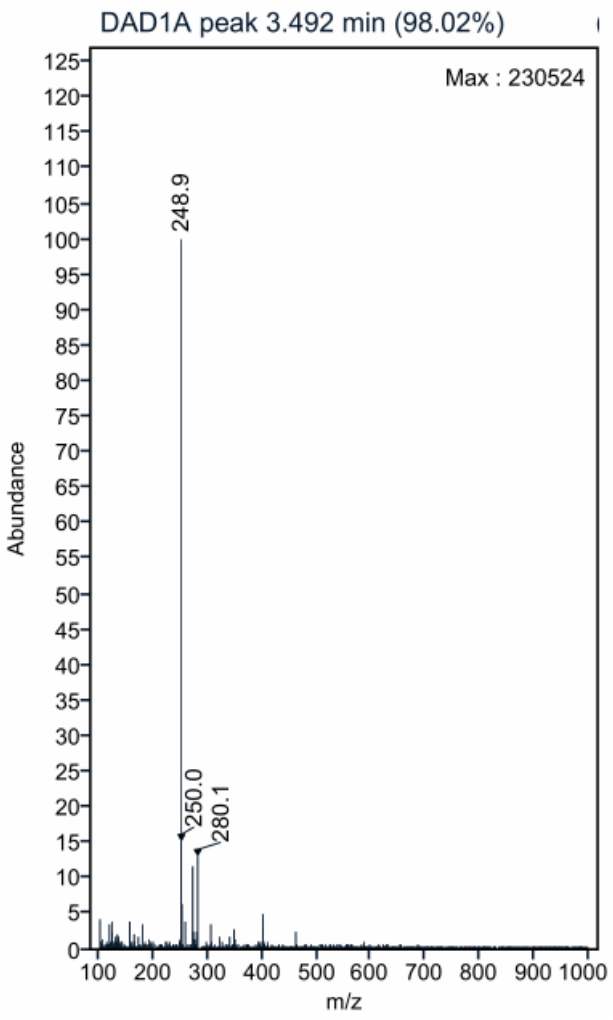


^{13}C NMR spectra of **TD19**

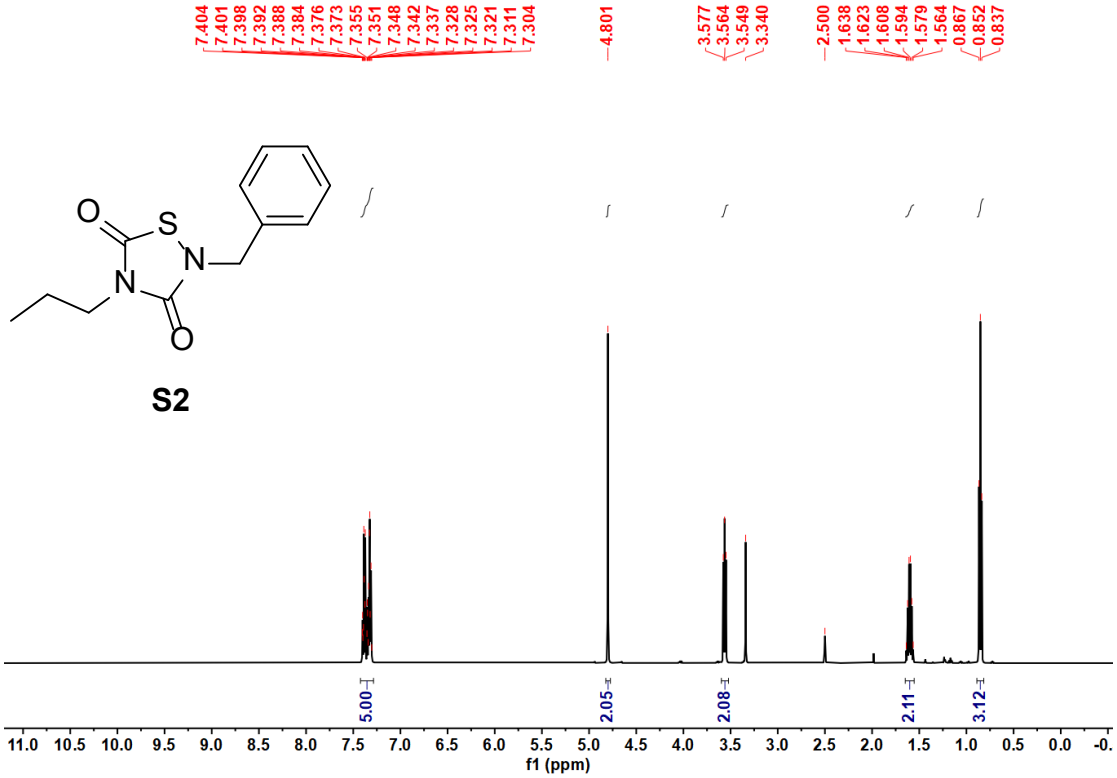


LC-MS spectra of **TD19** (purity, 98.02%)

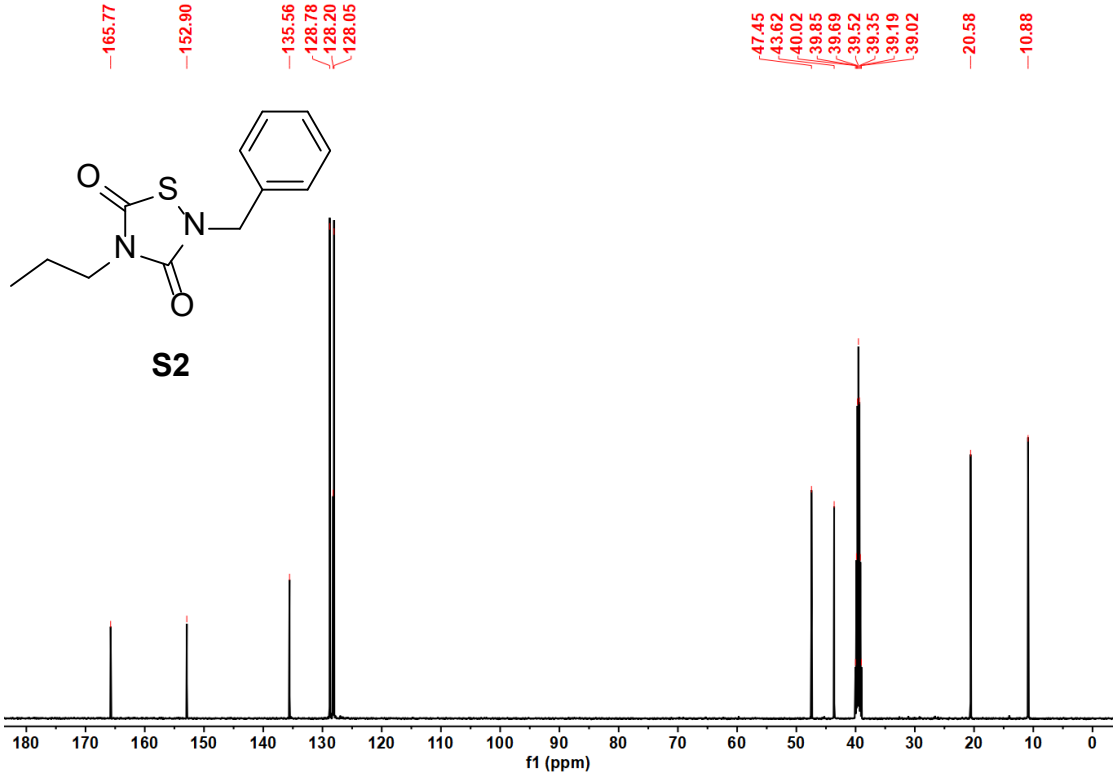




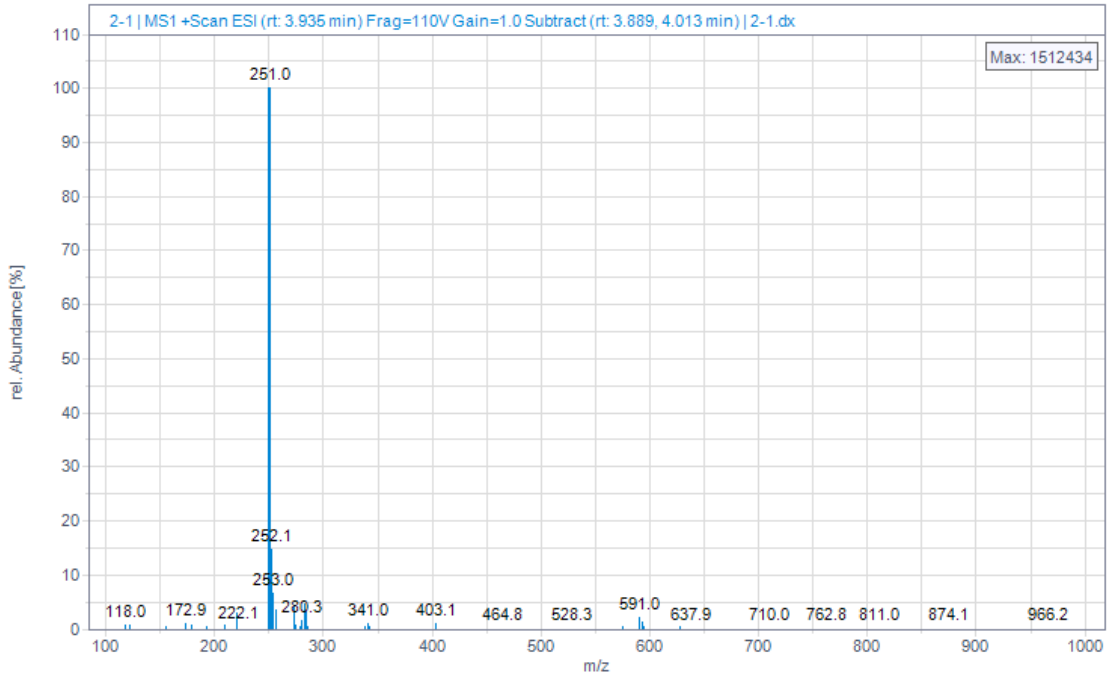
^{1}H NMR spectra of **S2**



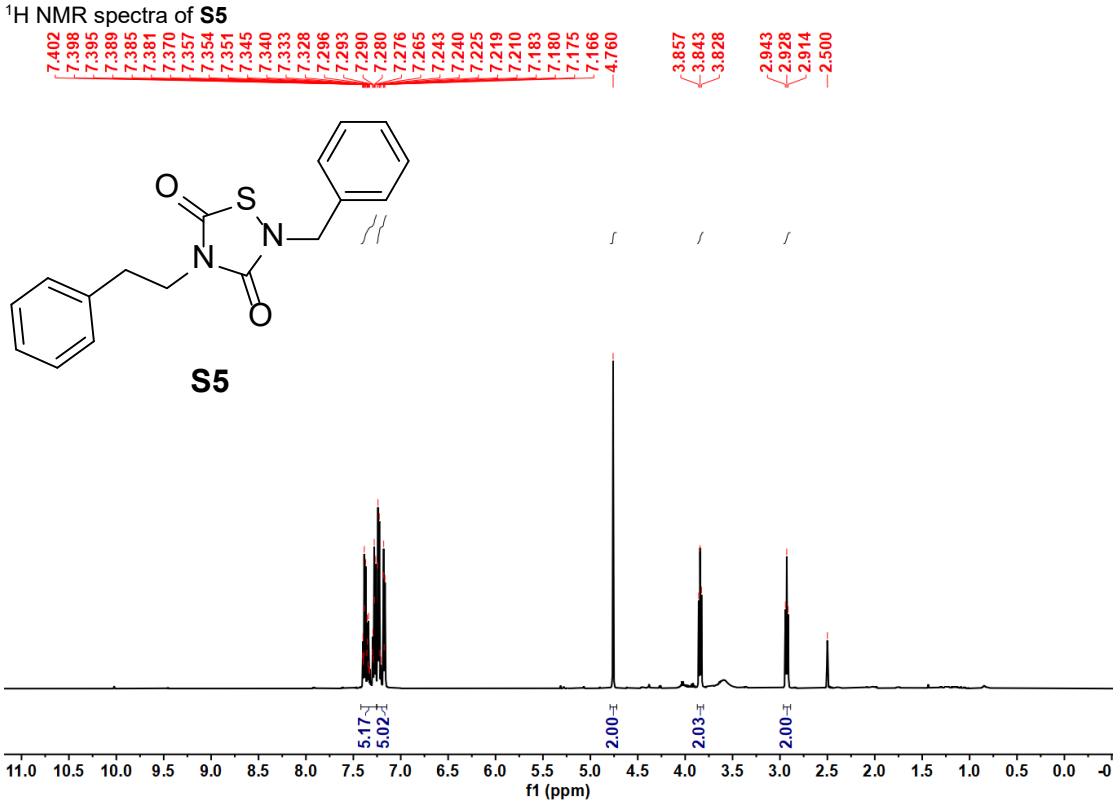
^{13}C NMR spectra of **S2**



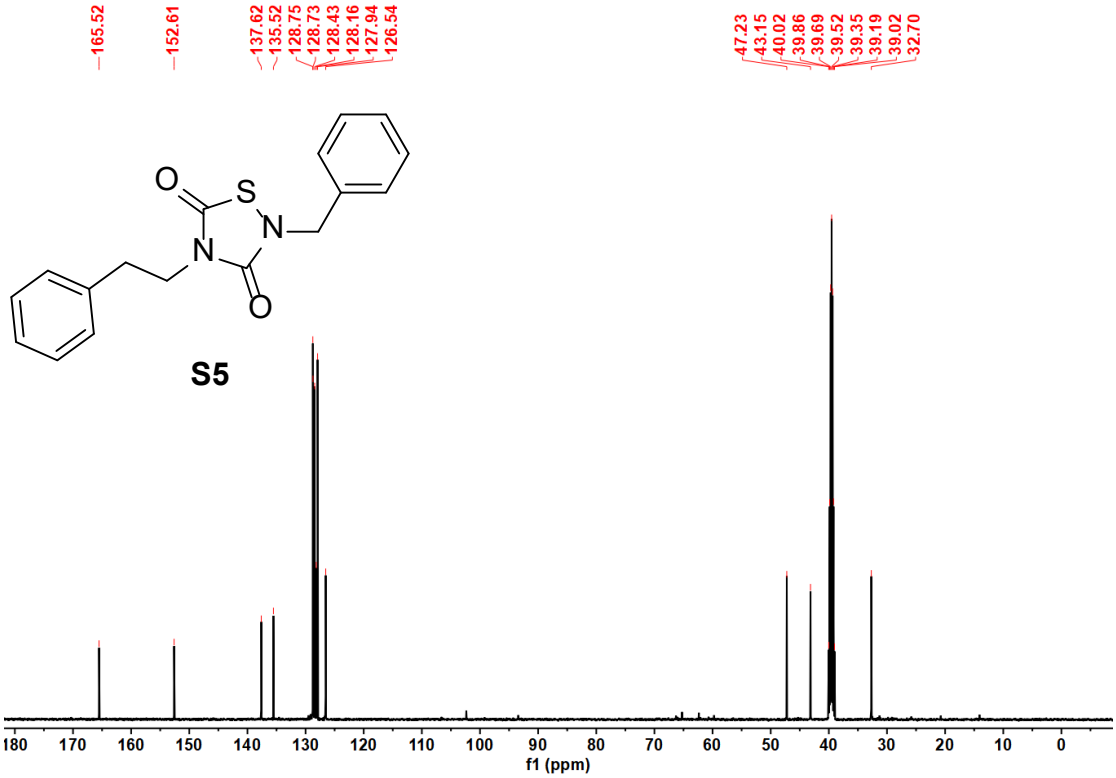
MS (ESI, positive ion mode) spectra of **S2**



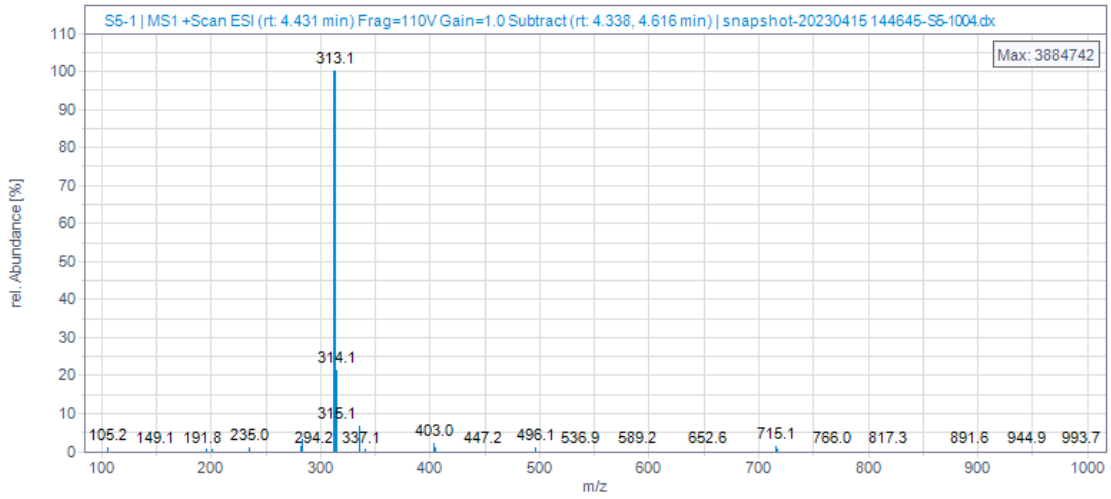
^{1}H NMR spectra of **S5**



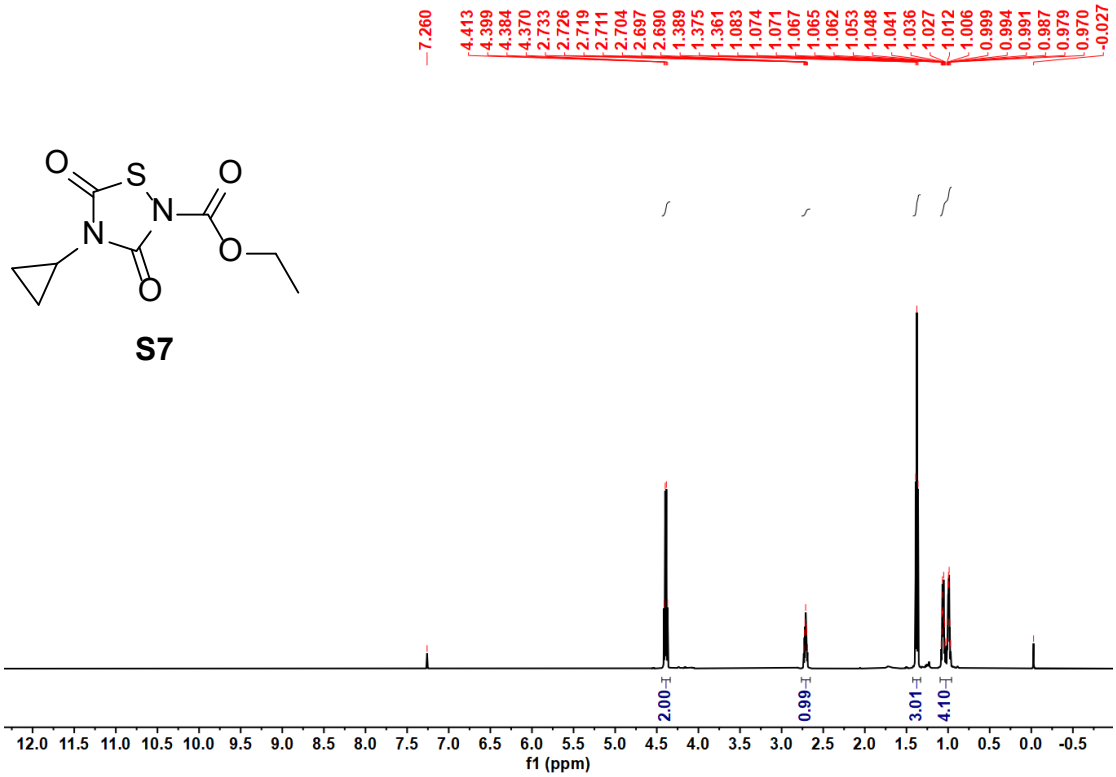
^{13}C NMR spectra of **S5**



MS (ESI, positive ion mode) spectra of **S5**

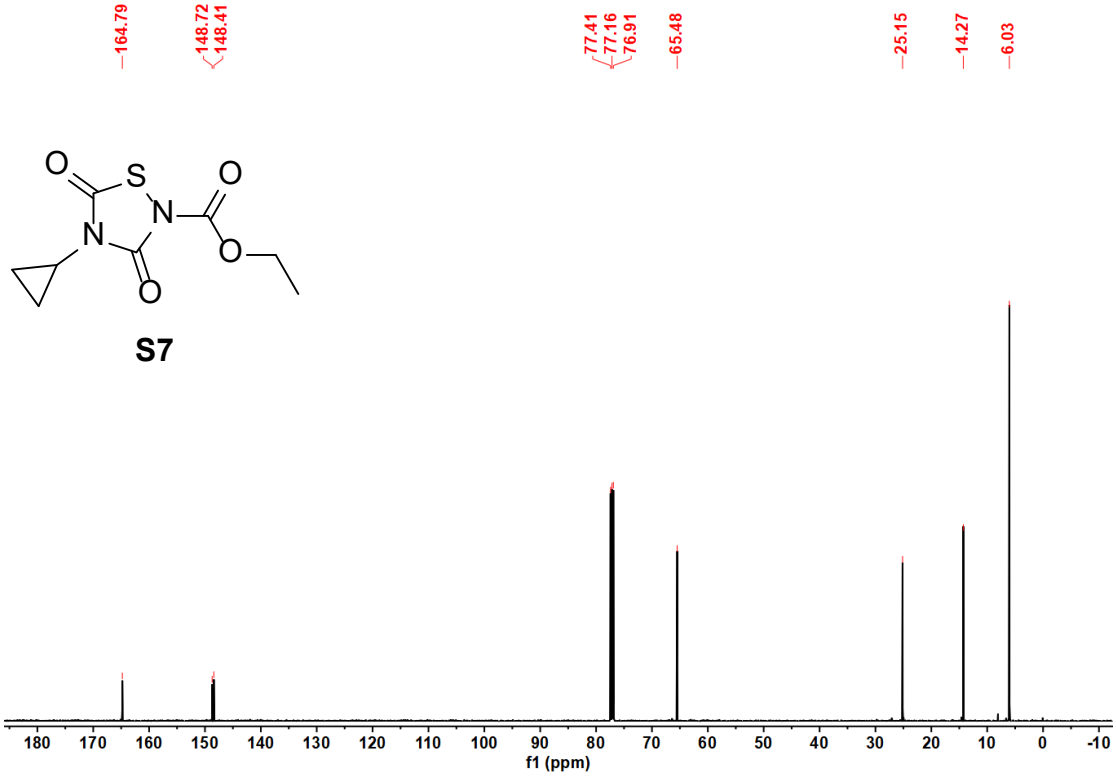


^{1}H NMR spectra of **S7**



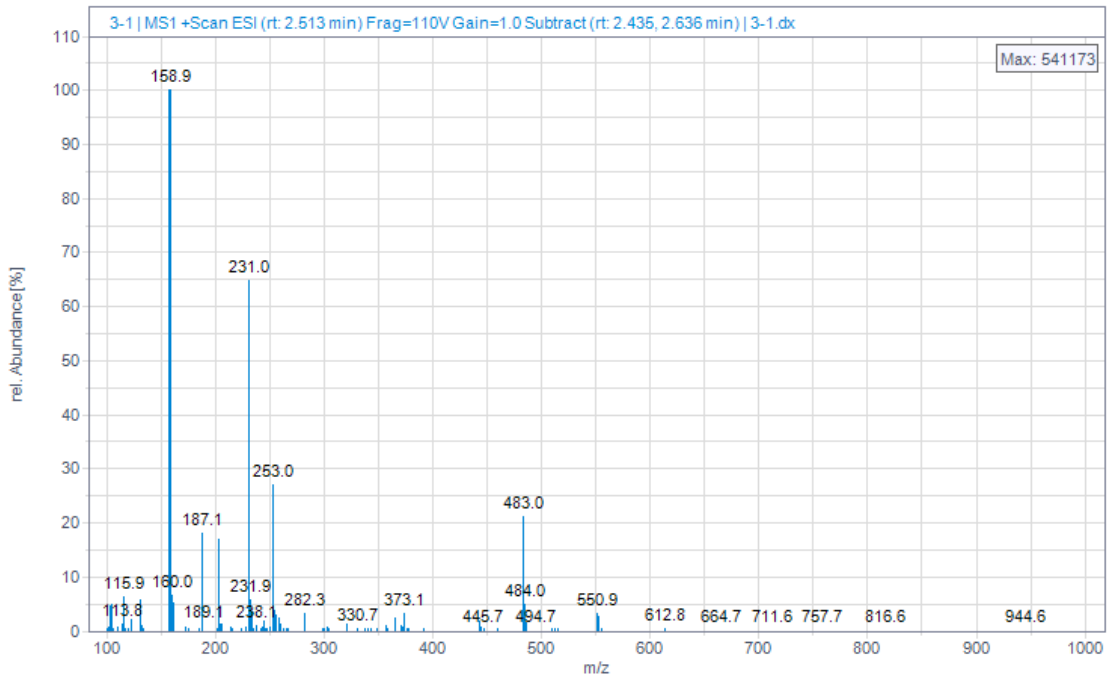
S7

^{13}C NMR spectra of **S7**

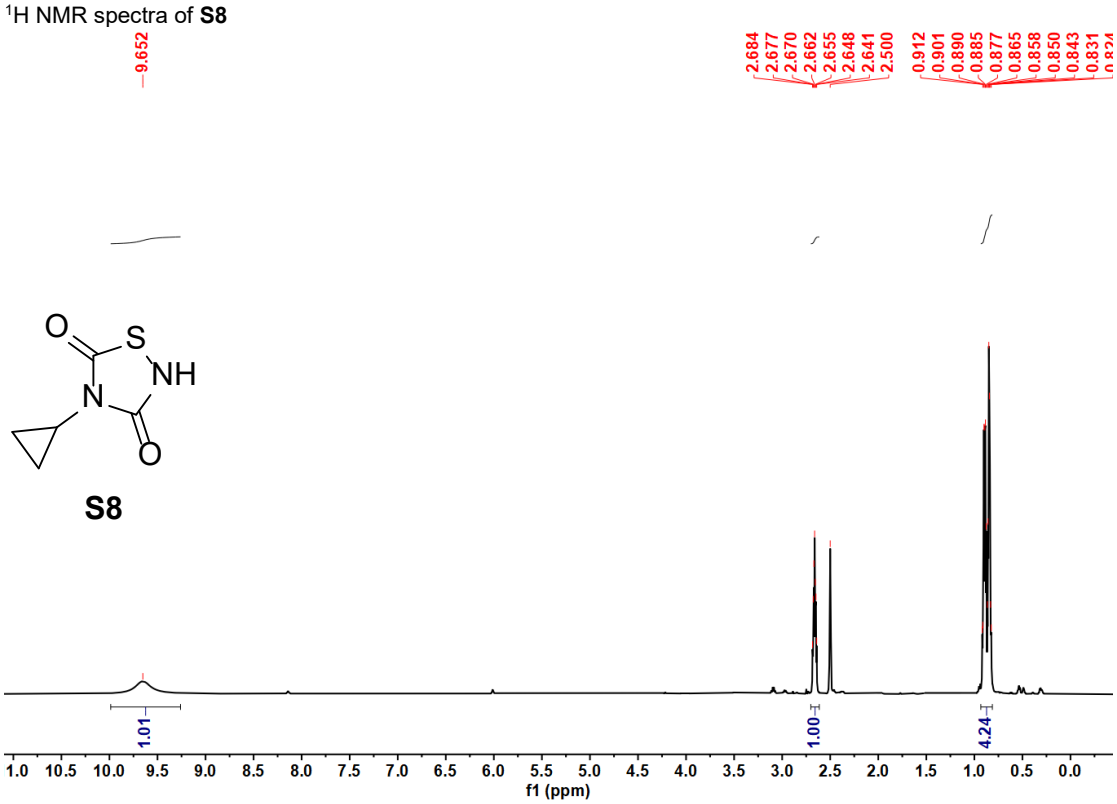


S7

MS (ESI, positive ion mode) spectra of **S7**

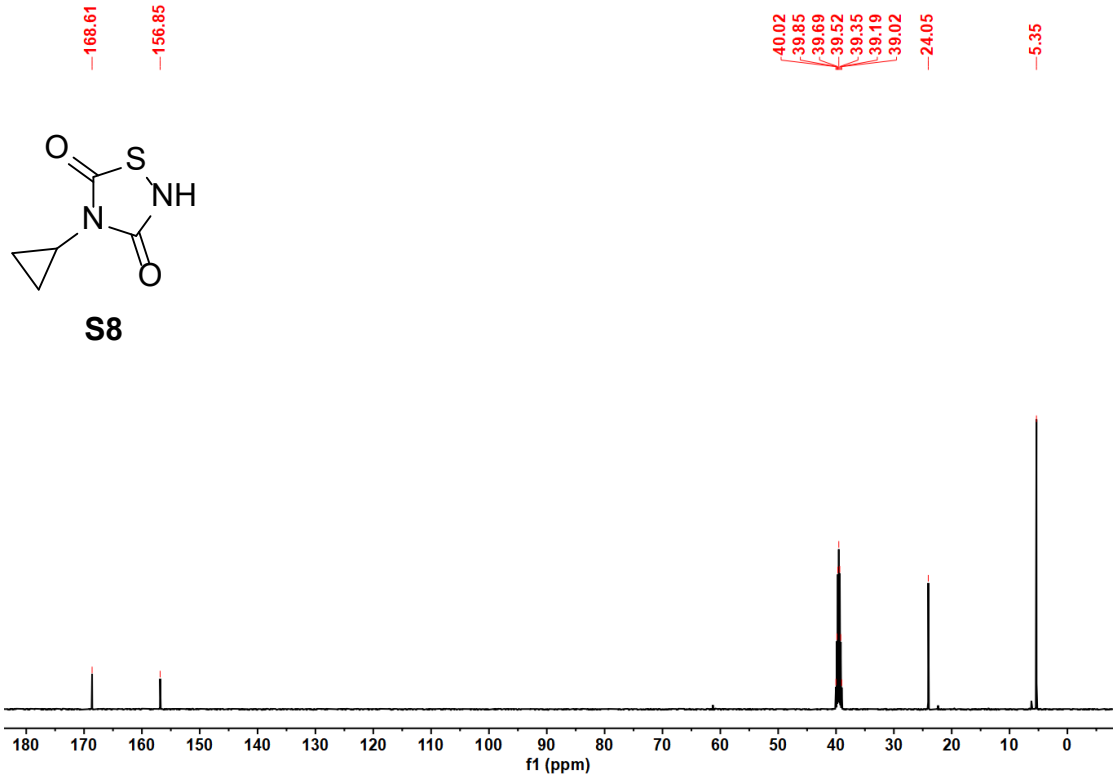


^{1}H NMR spectra of **S8**



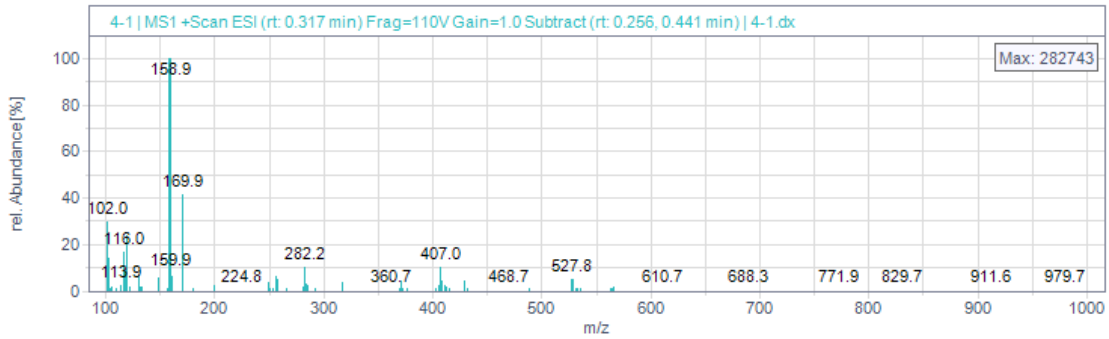
S8

^{13}C NMR spectra of **S8**

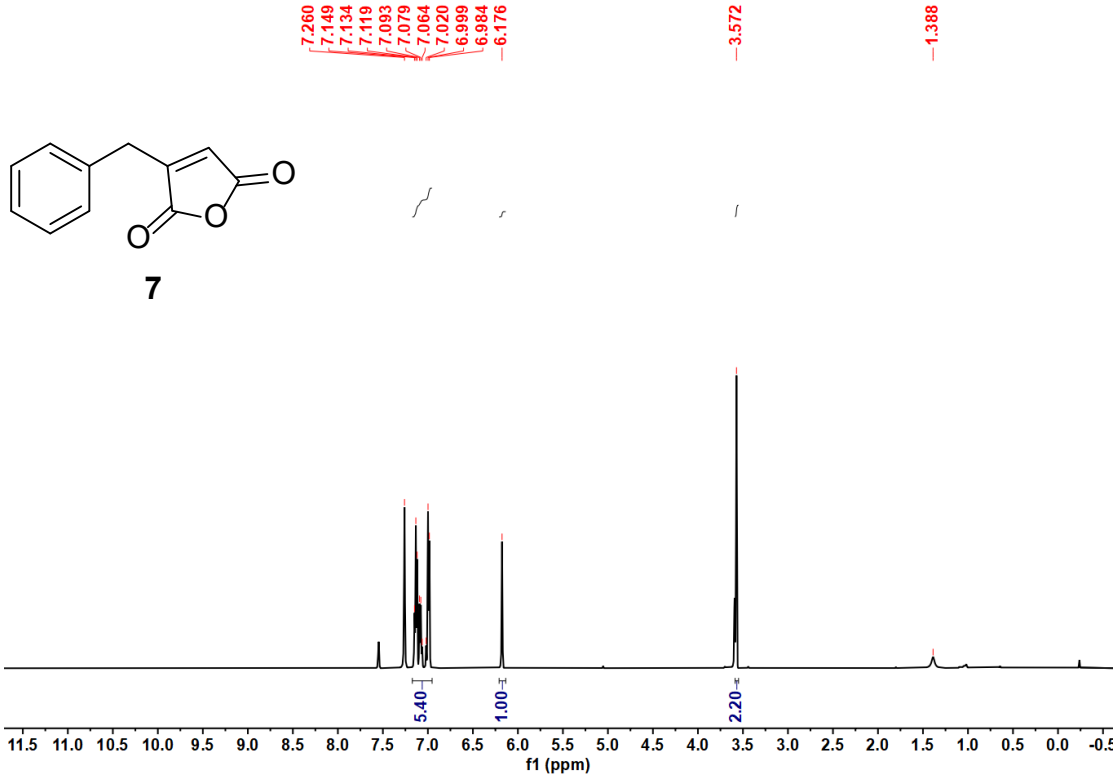


S8

MS (ESI, positive ion mode) spectra of **S8**

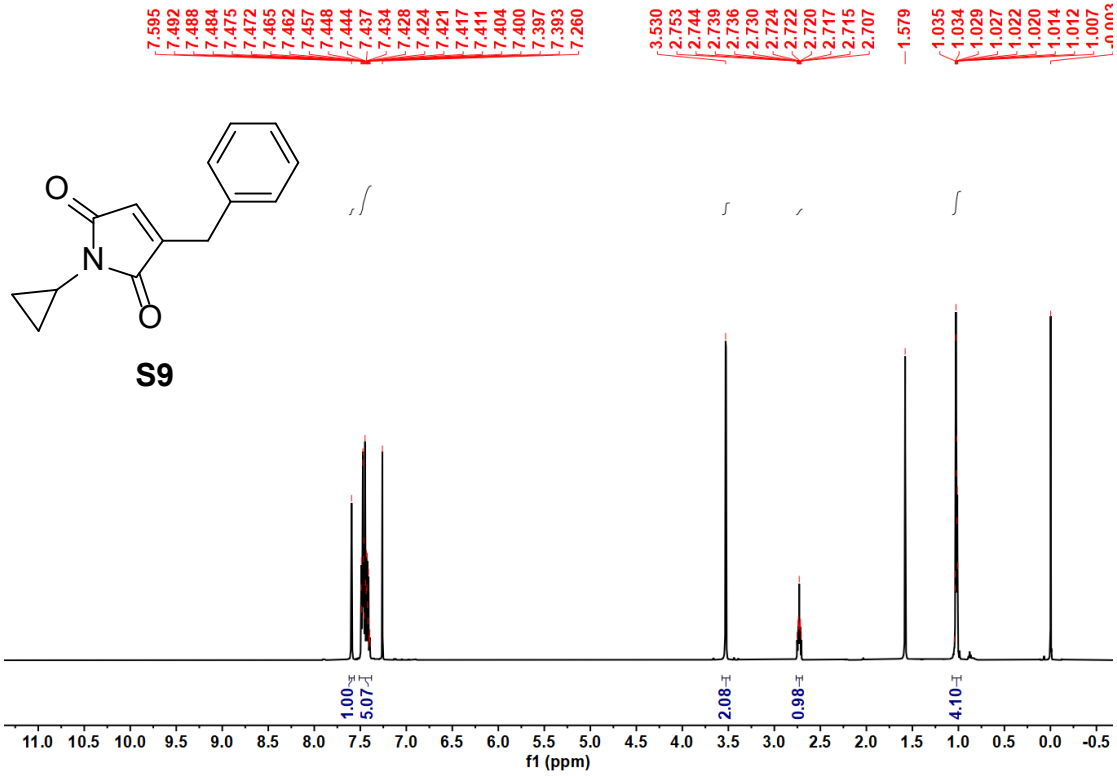


^{1}H NMR spectra of intermediate **7**



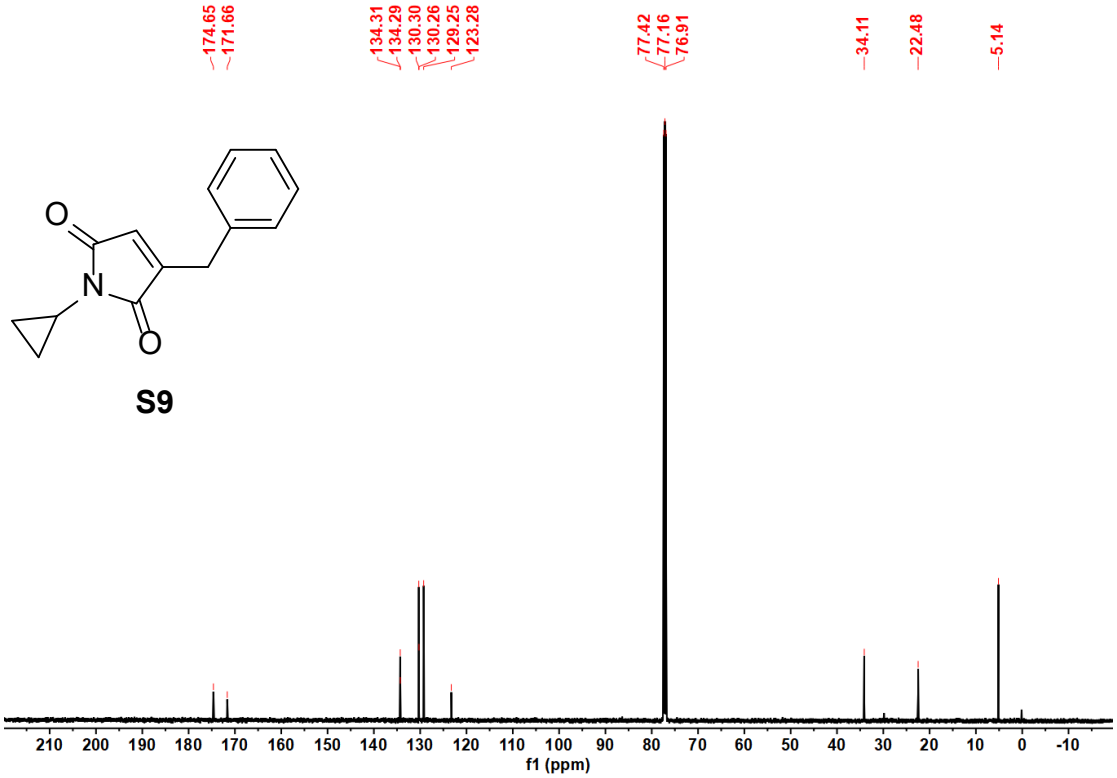
7

^{1}H NMR spectra of **S9**

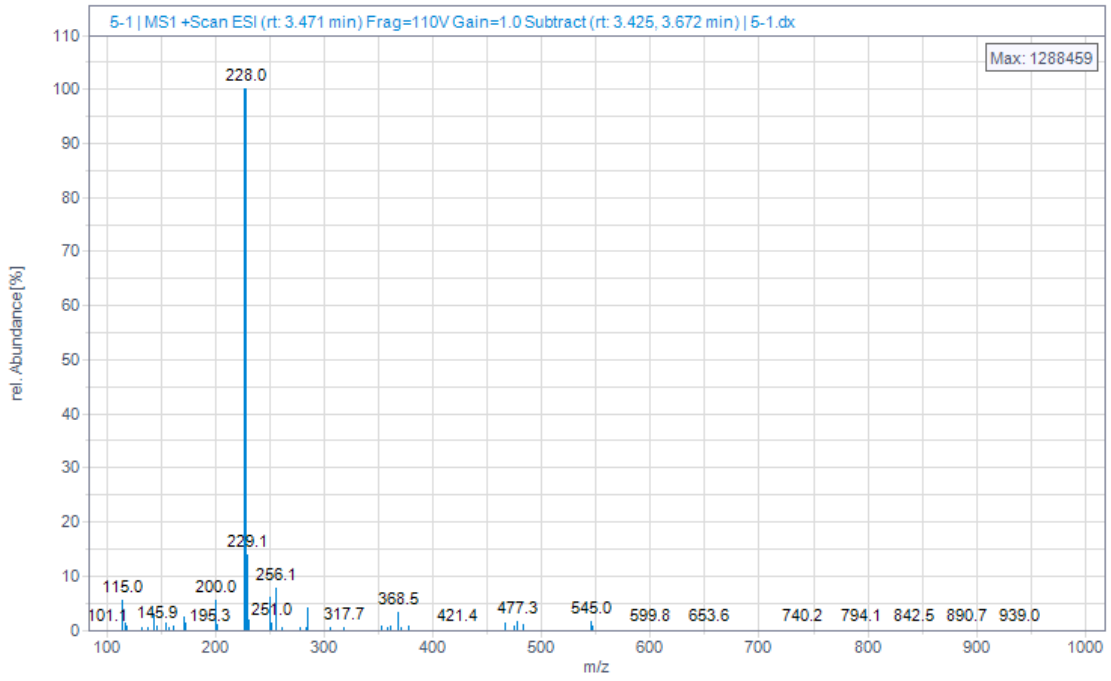


S9

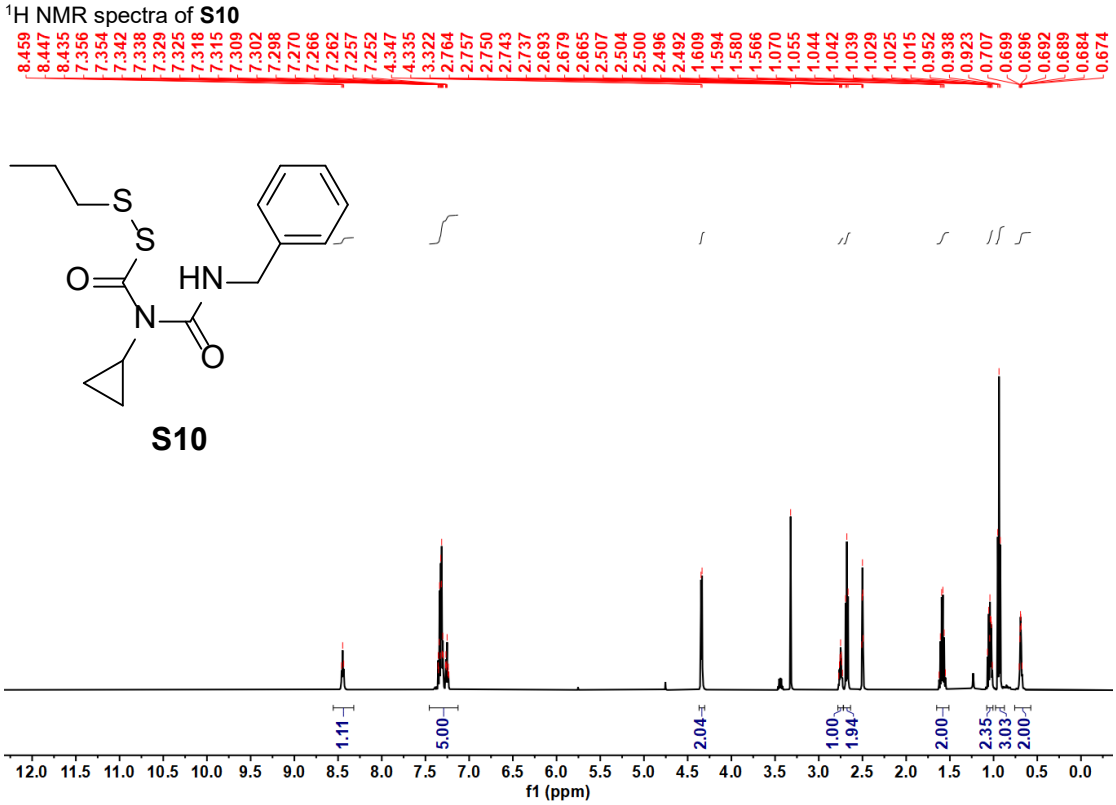
^{13}C NMR spectra of **S9**



MS (ESI, positive ion mode) spectra of **S9**

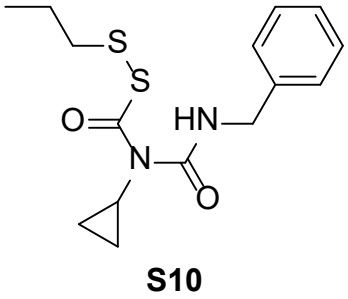


^{1}H NMR spectra of **S10**



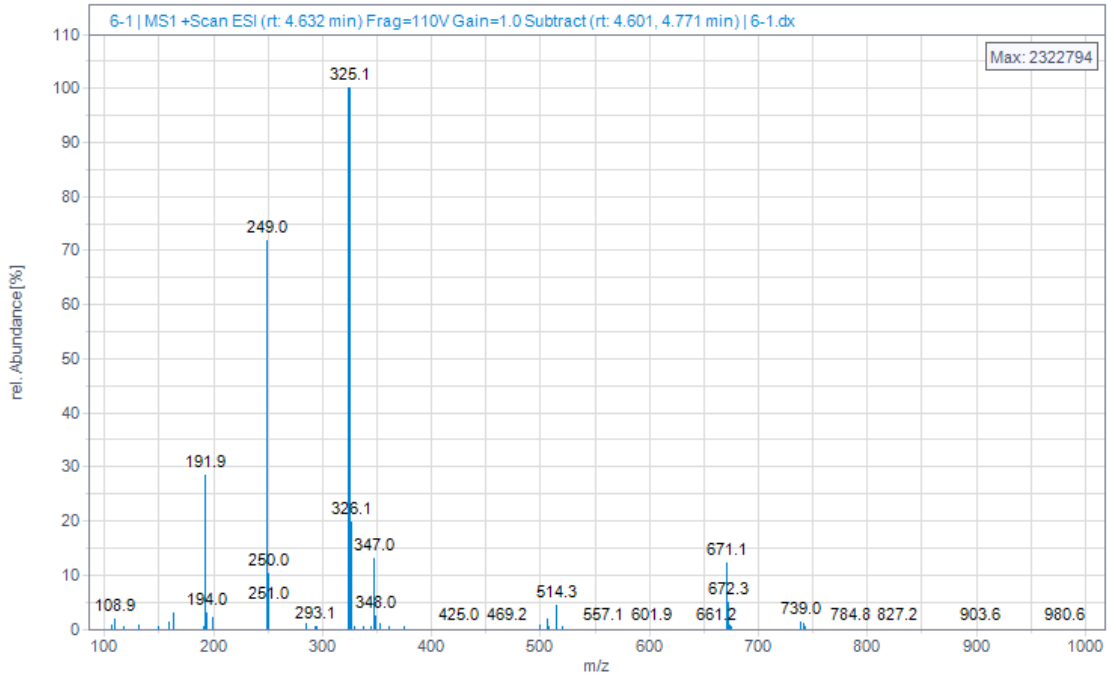
S10

^{13}C NMR spectra of **S10**



S10

MS (ESI, positive ion mode) spectra of **S10**



2. Protein expression and purification

The expression and purification of wild type or mutants of human ALKBH5$_{66–292}$ and zebrafish fALKBH5$_{38-287}$ were modified from previously reported methods.[2] In brief, wild-type and mutant gene sequences of the human ALKBH5$_{66-292}$ and zebrafish fALKBH5$_{38-287}$ were cloned into a modified pET28a (Novagen) vector containing a tobacco etch virus (TEV) protease recognition site located in the N-terminal region. Verification of the final clones was performed via DNA sequencing. The recombinated plasmids were transformed into *E. coli* strain BL21(DE3). The cells were cultured at at 37 °C in lysogeny broth (LB) medium containing 30 μg/mL kanamycin. Protein expression was induced at 16 °C for 16 h by adding 0.3 mM isopropyl-beta-D-thiogalactopyranoside once the A_{600} reached a range of 0.6-0.8. The cell pellets were collected after centrifuging the cultures at 5,000 g for 30 min, follwed by resuspension and sonication in lysis buffer (5% glycerol, 500 mM NaCl, 20 mM TrisHCl, pH 8.0). The cell lysates were cleared at 10,000 g for 1 h, and supernatant was loaded into a HisTrap™ HP column (GE Healthcare). The column was initially equilibrated with buffer A (20 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 8.0) and subsequently eluted with a gradient of buffer B (500 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 8.0). The eluate was treated with tobacco etch virus protease at a 1:10 (w/w, protease/proteins) ratio at 4 °C overnight to facilitate the removal of the His tag. To eliminate any remaining uncleaved proteins and tobacco etch virus protease, the eluate was subjected to a second HisTrap™ HP column. Additional purification was conducted in size exclusion chromatography using a Superdex 200 (GE Healthcare) column with buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl). The purity of peak fractions were examed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) an Coomassie Blue-stained. Finally, purified proteins were obtained for further bioassays.

The human FTO truncated with N-terminal 31 residues (FTO$_{\Delta N31}$) was expressed and purified as we described previously.[3] Briefly, *FTO*$_{\Delta N31}$ was cloned into pET28a (Novagen) vector. The recombinant plasmids were transformed into *E. coli* strain BL21(DE3). Cells were grown in LB medium with 30 μg/mL kanamycin at 37 °C. When the A_{600} reached a range of 0.6–0.8, Protein expression was induced by addition of 0.5 mM isopropyl-beta-D-thiogalactopyranoside, followed by culture at 16 °C for 16 h. Centrifuging the cultures and collecting the cell pellets. The cells were resuspended in lysis buffer (5% glycerol , 300 mM NaCl, 20 mM Tris-HCl, pH 8.0) and lysed by high pressure. The lysates were clarified by centrifuging at 10,000 g for 1 h, and the supernatant was applied to a HisTrap™ HP column (GE Healthcare). The column was equilibrited with buffer A (10 mM imidazole, 300 mM NaCl, 20 mM Tris-HCl, pH 8.0) and eluted by gredient buffer B (200 mM imidazole, 300 mM NaCl, 20 mM Tris-HCl, pH 8.0). The fractions were collected and loaded into a MonoQ column (GE Healthcare), and eluted by gredient buffer (500 mM NaCl, 20 mM Tris-HCl, pH 8.0), followed by gel filtration in the Superdex 200 (GE Healthcare) coloumn. The purified proteins were pooled and concentrated to 20 mg/ml, and stored at -80 °C.

The full length *ALKBH3* gene was cloned into pET28a vactor. The recombinant plasmids were transformed into *E. coli* strain BL21(DE3). The cells were grown in LB medium at 37 °C until the A_{600} reached a range of 0.6-0.8. The expression of the ALKBH3 protein was induced by adding 1 mM isopropyl-beta-D-thiogalactopyranoside and incubating the cells at 16 °C for an additional 16 h. The cells were then harvested by centrifugation at 5,000 g for 30 min, and the resulting pellets were resuspended in lysis buffer (5% glycerol, 300 mM NaCl, 20 mM Tris-HCl, pH 8.0) and lysed by high pressure. After centrifugation at 10,000 g for 1 h, the supernatant was loaded onto a HisTrap™ HP column (GE Healthcare) equilibrated with buffer A (10 mM imidazole, 300 mM NaCl, 20 mM Tris-HCl, pH 8.0). The protein was eluted using a gradient of buffer B (200 mM imidazole, 300 mM NaCl, 20 mM Tris-HCl, pH 8.0). The eluted fractions were pooled and concentrated to 20 mg/mL, and stored at -80 °C for further bioassays.

3. PAGE-based assay

To assess the enzyme activity of m^{6}A demethylases, including ALKBH5 and FTO, we performed a polyacrylamide gel electrophoresis (PAGE) based assay modified from previously reported methods.[4] The 39 nt ssDNA substrate [5'-ATTGCCATTCTCGATAGG(dm^{6}A)TCCGGTCAAACCTAGACGAA-3'], which featured the G(dm^{6}A)TC sequence, underwent demethylation catalyzed by ALKBH5/FTO, resulting in the generation of the GATC sequence without dm^{6}A modification. The assay reaction mixtures comprised 50 mM Tris-HCl pH 7.5, 1 μM single-stranded DNA (ssDNA), 1 μM FTO, or 2 μM ALKBH5, along with 300 μM 2-oxoglutarate (2OG), 280 μM $(NH_4)_2Fe(SO_4)_2$, and 2 mM L-ascorbic acid. Additionally, varying concentrations of the compounds were included in the reaction mixtures. Subsequently, the single-stranded DNA was annealed to its complementary strand [5'-TTCGTCTAGGTTTGACCGGATCCTATCGAGAATGGCAAT-3'], forming a double-stranded DNA with an unmethylated GATC sequence, which can be specifically cleaved by the DpnII enzyme. Upon DpnII digestion, the unmethylated 39 bp double-stranded DNA was cleaved, yielding 22 bp and 17 bp fragments, while the methylated counterpart could not be cleaved. The digestion samples were subjected to analysis using a 15% non-reducing polyacrylamide gel electrophoresis (PAGE), and the intensity of the bands was measured through Gel-Red staining. The uncleaved 39 bp samples were observed in the upper band, while the 22 bp and 17 bp fragments were not totally separated in the gel and were present in the lower band. The enzyme activity of ALKBH5/FTO was quantified by calculating the percentage of lower 22 bp and 17 bp fragments. The PAGE-based assay for ALKBH3 was conducted in a similar manner to that of ALKBH5 and FTO, with the exception that the 39 nt ssDNA substrate used in the assay contained a dm^{1}A modification instead of dm^{6}A. For ALKBH5 and ALKBH3, the positive control was the compound **rhein**, while for FTO, the positive control was **FB23**.

4. Isothermal titration calorimetry (ITC)

ITC experiments were conducted using a MicroCal PEAQ-ITC system (Malvern Panalytical) in a buffer containing 1×PBS pH 7.4, 1% DMSO at 25 °C with constant stirring at 750 rpm, following the previously reported method.[5] The proteins and compounds were dissolved in the same buffer, and the cell was filled with 50 μM of either ALKBH5 or FTO, while the syringe contained **TD19** at 500 μM or m^{6}A-RNA substrate (5'-UGG(m^{6}A)CUGC-3') at 1 mM. After automatic equilibration of the system, 2 μL of solution in the syringe was injected into the cell to trigger the binding reaction and produce a characteristic peak sequence in the recorded signal. Data analysis, including baseline correction and evaluation, was carried out using MicroCal PEAQ-ITC Analysis Software provided. Fits were carried out considering all injections except the first injection and outliers to calculate the maximum heat and the equilibrium dissociation constant (K_d) value.

5. Microscale thermophoresis (MST)

The purified recombinant proteins were dialyzed into the buffer conatined 1×PBS pH 7.4, and 0.05 % Tween 20 and then labeled with a red fluorophore following the Monolith His-Tag Labeling Kit RED-tris-NTA protocol (MO-L018, Nanotemper). All tested stock compounds were serially diluted into the same buffer (1×PBS pH 7.4 and 0.05 % Tween 20) with the final DMSO concentration of 0.25% for the MST assay. Monolith NT.115 instrument (NanoTemper Technologies) was used to perform the MST experiment. Labeled proteins (20 nM) were mixed with the indicated concentrations of candidate compounds in the reaction buffer containing 1×PBS pH 7.4 and 0.05 % Tween 20. The MST data were collected using 100% excitation power and 40% MST power, and analyzed by Nanotemper analysis software (MO.Affinity Analysis v2.3).

6. FRET-based m^{6}A detection assay

The FRET-based m^{6}A detection assay was adapted from a previously reported method.[6] In brief, in 30 μl reaction voloum, 1 μM "(m^{6}A)CA" chimera probe (5'-FAM-d(CAT)r(GG(m^{6}A)CA)d(TATGT)-3'-dual-BHQ-1, synthesis by Shanghai Generay Biotech Co., Ltd., China) were demethylated by 0.5 μM ALKBH5 in the demethylated buffer (300 μM 2OG, 140 μM $Fe(NH_4)_2(SO_4)_2$, 2 mM L-ascorbic acid, and 50 mM Tris-HCl pH 7.5). After incubating at room temperature for 60 min, the reaction was heat-inactivated at 65 °C for 5 min. 1/10 volume of the sample was mixed with (0.05 U/μL) MazF (2415A, Takara) in the buffer containing 0.01% Tween 20, 5 mM EDTA, 40 mM sodium phosphate pH 7.5. The mixture was loaded onto a 384-well nonbinding surface (NBS) microplate (3575, Corning), and the fluorescences (Ex. 485 nm, Em. 535 nm) were monitored in SPARK multifunctional microplate reader (TECAN) at 37 °C every 60 seconds for 30 min. The saturated intensities of fluorescences were used for further analysis. In the time-dependent inhibition assays, different concentrations of **TD19** were pre-incubated with ALKBH5 for various durations. The reactions were initiated by adding the "(m^{6}A)CA" chimera probe follwed by fluorescence detection. The fluorescence intensity of the sample treated with DMSO was used as a reference to calculate the percentage inhibition of enzymatic activity by the compounds. The IC_{50} values were then calculated by plotting the percentage inhibition against the logarithm of the compound concentrations.
In order to ascertain the k_{inact} and K_I values, a series of variable inhibitor concentrations were incubated with ALKBH5 for varying durations, and the resulting enzyme kinetics curves were constructed. The natural logarithms of the remaining relative activity of ALKBH5 were plotted against the incubation time, followed by a linear regression analysis to determine the first-order rate constants (K_{obs}). These K_{obs} values were subsequently plotted against inhibitor concentration and fitted to the ensuing equation to obtain the k_{inact} and K_I values:[7]

$$K_{\mathrm{obs}} = \frac{k_{\mathrm{inact}} * [\mathrm{I}]}{K_{\mathrm{I}} + [\mathrm{I}]}$$

7. CPM labeling assay

The ALKBH5 and fALKBH5 proteins were diluted to a concentration of 10 μM in a buffer solution consisting of 50 mM Tris (pH 7.5) and 150 mM NaCl, and was then incubated with 100 μM **TD19** for a duration of 60 min. At each time point, 27 μL of the sample was combined with 3 μL of CPM that was previously dissolved in DMSO (at a final concentration of 100 μM) within a black 384-well plate. The fluorescence of the samples was measured at 384/470 nm, while the sample treated with DMSO was utilized as a reference for determining the CPM labeling ratio.

8. Intact protein mass detection
Protein sample solution was diluted to a concentration of 1 mg/mL in water with 0.1% formic acid. A total of 2 μg of ALKBH5 protein was injected for each LC/MS run.

9. Drug conjugate sites analysis

100 μg of sample was precipitated by six volumes of acetone at −20 °C for 30 min and centrifuged at 15,000 g for 10 min to remove unbound drug compounds. Precipitated proteins were dried in air and resuspended in 100 mM ammonium bicarbonate. Sequencing-

grade modified trypsin was added to each sample (enzyme to protein ratio 1:25, w/w) and incubated at 37 °C for 16 h. 20 μg of the digested peptide mixture was desalted by C18 tip as previously described.[8]

10. LC/MS instruments and bioinformatics

Intact protein was analyzed using an Agilent 1290 Infinity II LC coupled with a 6545-XT QTOF mass spectrometer (Agilent, Santa Clara, CA, USA). Intact protein samples were separated with an Agilent PLRP-S column (1.0 X 50 mm, 5 um) using a 15 min gradient (hold at 5% B for 5 min, 5-95% B for 5 min, 95% hold for 2 min, 95%-5% B for 0.1 min, 5% B for 2.9 min) at a flow rate of 0.300 mL/min. Mobile Phase A was made up of water with 0.1% formic acid, while Mobile Phase B was made up of acetonitrile with 0.1% formic acid. The mass spectrometry instrument parameters were set as the following: the dry gas flow rate was set 10.0 L/min at 325 °C, the nebulizer was set at 40 psig, the capillary voltage was set at 3.5 kV and the scan range was from 350 to 3,000 m/z at 1 Hz.
Enzyme digests of peptide mixture were analyzed on the Easy nano-LC1000 system using a self-packed column (75μm × 150mm; 3 μm ReproSil-Pur C18 beads, 120 Å , Dr.Maisch GmbH, Ammerbuch, Germany) at a flow rate of 300 nL/min. The mobile phase A of RP-HPLC was 0.1% formic acid in water, and B was 0.1% formic acid in acetonitrile. The peptides were eluted using a gradient (2–90% mobile phase B) over a 60min period into a nano-ESI orbitrap Fusion mass spectrometer. The mass spectrometer was operated in data-dependent mode with each full MS scan (m/z 350-1800) followed by MS/MS for the 15 most intense ions with the parameters: ≥ +2 precursor ion charge, 2 Da precursor ion isolation window and 35 normalized collision energy of HCD. Dynamic Exclusion™ was set for 30 s. The full mass and the subsequent MS/MS analyses were scanned in the Orbitrap analyzer with R = 60,000 and R= 15,000, respectively.
The LC/MS raw data was processed using MassHunter BioConfirm (Version 10.0, Agilent, Santa Clara, CA, USA) to deconvolution the Intact protein masses.
The LC/MS raw data for peptide analysis was processed using Maxquant with a false discovery rate (FDR) < 0.01 at the levels of peptides and proteins. Trypsin/P was selected as the digestive enzyme with two potential missed cleavages. The search included variable modifications of methionine oxidation and N-terminal acetylation, and cysteine drug conjuction (+248.30).

11. Pull-down assay

Pull-down assays were performed according to a previously reported method.[9] In brief, the assays were conducted in a total volume of 100 μl containing 20 μl of streptavidin agarose (20353, Thermo Scientific), 5 μM biotinylated DNA substrate (5′-biotin-TEGTTTTTTGG(m^6A)CT-3′, purchased from GenScript), 25 μM of either wild-type or mutant ALKBH5 proteins, varying concentrations of **TD19**, and 500 μM 2OG. The mixture was rocked at 4 °C for 30 min, and spun at 12,000 g for 1 min, after which the supernatant was discarded. The precipitates were washed three times with H_2O to eliminate any residual unbound protein, and then resuspended in 30 μl of SDS loading buffer. The samples were boiled for 10 min, spun at 12,000g for 30 s, and 10 μl of the supernatant was loaded onto a Hepes-Tris 4-12% Precast Protein Plus Gel (36255ES10, Yeasen). The gels were stained using Coomassie-brilliant blue.

12. Cell cultures

NB4 and MOLM13 cells were cultured in RPMI 1640 medium, supplemented with 10% fetal bovine serum (FBS) (Gibco), antibiotics. U87 and A172 cells were cultured in MEM medium, supplemented with 10% FBS (Gibco) and antibiotics. The cells were incubated at 37 °C in an atmosphere of 5% CO_2. The cell lines were validated to be devoid of mycoplasma contamination.

13. Cell proliferation assay

To evaluate the effect of **TD19** on cell proliferation, AML cells were seeded at a density of 8000 cells/well, whereas GBM cells were seeded at a density of 3000 cells/well, and treated with either **TD19** or DMSO for 48 h. The proliferative capacity of both cell types was determined by adding 10 μL of Cell Counting Kit-8 reagent (MA0218, Meilunbio) to each well for AML cells, or 10 μL of Methylthiazolyldiphenyl-tetrazolium bromide (MB4698-1, Meilunbio) for GBM cells, and incubating the cells at 37 °C in 5% CO_2 for 4 h. The absorbance was measured at 450 nm for AML cells, and at 490 nm for GBM cells, using a microplate reader. The experiment was repeated thrice for each compound concentration, and data were analyzed using GraphPad Prism software.

14. Western blot assay

The experimental details were reported previously.[10] Antibodies to ALKBH5 (ab195377, Abcam), FTO (ab126605, Abcam), and GAPDH (G8795, Sigma) were procured from commercial sources. The cells were treated with either DMSO or **TD19** at varying concentrations for 48 h. Following this, cells were washed twice with chilled PBS and suspended in RIPA lysis buffer (P0013C, Beyotime). The lysate was centrifuged at 12,000 rpm for 20 min, and the protein content was quantified via the bicinchoninic acid

(BCA) assay (P0011, Beyotime). Subsequently, the protein was separated on 10% SDS-PAGE and transferred onto a nitrocellulose membrane (Millipore, USA), which was blocked using 5% skim milk. The membrane was then incubated with the primary antibody at 4 °C overnight, followed by washing with TBST buffer and incubation with a secondary antibody for 1 h at room temperature. Finally, the membrane was visualized using the ECL detection system (GE Healthcare Bioscience, USA).

15. The drug affinity responsive target stability (DARTS) assay

The DARTS assay was performed in AML or GBM cells with some modifications based on a previously published protocol.[11] In brief, NB4 or U87 cells were collected and washed with chilled PBS. The cells were then lysed in M-PER buffer (78501, ThermoFisher) supplemented with protease inhibitor cocktail (C600387, Sangon Biotech) and phosphatase inhibitor cocktail (78420, ThermoFisher). The cell lysates were kept on ice for 10 min and then subjected to centrifugation at 12,000 g for 20 min at 4 °C. The resulting supernatant was mixed with an appropriate volume of 10 × TNC buffer to make a final concentration of 1× TNC buffer (50 mM Tris-HCl (pH 8.0), 50 mM NaCl, and 10 mM CaCl2) in the lysate. a BCA assay (P0012, Beyotime) was performed to determine the concentration of protein. Next, the cell lysates were incubated with varying concentrations of **TD19** as well as DMSO as a negative control for 30 min at room temperature. The lysates were then digested with pronase (10165921001, Roche) for 10 min, and the reaction was quenched by adding protease inhibitor cocktail. Finally, the cell lysates were analyzed by western blot to determine the abundance of ALKBH5 or FTO.

16. m^{6}A dot blot assay

The cells were collected and their total RNA was isolated using the total RNA kit (DP419, Tiangen) following the manufacturer's instructions. The RNA samples were diluted in RNAase-free water and denatured at 95 °C for 5 min, followed by immediate cooling on ice. The samples were then spotted onto a Hybond-N+ membrane (RPN119B, GE Healthcare) using a Bio-Dot Apparatus (170-6545, Bio-Rad) and cross-linked to the membrane using UV irradiation. The membrane was washed with PBST and blocked with 5% non-fat powdered milk (A600669, Sangon Biotech) for 1 h at 25 °C, and then incubated with an m^{6}A rabbit polyclonal antibody (AF7407, Beyotime) overnight at 4 °C. The membrane was then incubated with an HRP-conjugated goat anti-rabbit IgG antibody (A0208, Beyotime) and developed using an ECL detection reagent (4TK1539, Tanon) for immunoblotting. The membrane was subsequently subjected to staining with 0.02% methylene blue, serving as a loading control.

17. Lentivirus production and infection

To achieve knockdown and overexpression of ALKBH5 in AML and GBM cells, lentivirus-mediated gene silencing was performed with some modifications to a previously reported protocol.[12] To package the lentivirus, a total of 1.5 μg of pMD2.G (12259, addgene), 2.1 μg of pMDLg/pRRE (60488, addgene), and 0.9 μg of pRSV-Rev (12253, addgene), along with 5.4 μg of either pLVX-IRES-Neo-oeALKBH5, pLKO.1-shALKBH5 (shA5-1: TRCN0000064787; shA5-2: TRCN0000064786), or control vectors, were co-transfected into 293T cells in 100 mm cell-culture dishes using Lipofectamine™ 2000 transfection reagent (11668030, Invitrogen). The lentivirus particles were harvested at 48 h and 72 h post-transfection, and were then directly added into cells, along with 4 μg/ml polybrene. To select for positively infected cells, 1 μg/ml puromycin was added to the cultured cells 48 h after transfection.

18. Quantitative RT-qPCR

For quantitative RT-qPCR assay, total RNA was isolated using total RNA kit (DP419, Tiangen) and subjected to reverse transcription using the HiScript III RT SuperMix for qPCR Kit (R323-01, Vazyme). RT-qPCRs were performed using ChamQ Universal SYBR qPCR Master Mix (Q711-02/03, Vazyme) with a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Gene expression was quantified utilizing the comparative ΔΔCT method, whereby GAPDH expression was utilized as a normalization control. The following primers were used:
GAPDH: F-5'-GCCGCATCTTCTTTTGCGTC-3',
R-5'-TGAAGGGGTCATTGATGGCA-3'.
ALKBH5: F-5'-CGGCGAAGGCTACACTTACG-3',
R-5'- CCACCAGCTTTTGGATCACCA-3'.
AXL: F-5'- CCGTGGACCTACTCTGGCT-3',
R-5'- CCTTGGCGTTATGGGCTTC-3'.
FOXM1: F-5'-CTATACGTGGATTGAGGACC-3',
R-5'-GAATGGTCCAGAAGGAGACC-3'.

19. Covalent docking procedures

The crystal structure of human ALKBH5 in complex with 2-OG (PDB ID: 4NRO) was obtained from RCSB PDB database and used for the modelling of binding modes. The protein structure was prepared using Schrödinger 2018, which added hydrogens, assigned bond orders, and removed waters. Compound **TD19** was built in ChemDraw 20.0 and prepared in LigPrep module. The grid box was centered on the selected cysteine residues. After preparations, the docking program for **TD19** was performed in Covalent Docking module, the reaction type of disulfide formation was selected. The best poses with low energy conformations were selected and proceeded in PyMOL Molecular Graphics Systems (version 2.5.8).

Supplemental Figures

Fig. S1

a

Residues	100	200	227	230	267
M. Musculus mouse ALKBH5	C	C	C	C	C
D. rerio zebrafish Alkbh5	C	C	C	C	C
H. Sapiens FTO	S	A	G	I	C
H. Sapiens ALKBH2	G	H	V	H	S
H. Sapiens ALKBH3	V	S	E	K	R

b

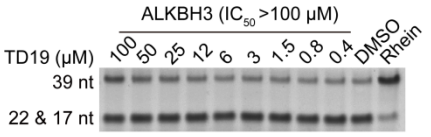


Fig. S1. Development of **TD19** as a selective ALKBH5 inhibitor. (a) A partial sequence alignment of cysteines in human ALKBH5 with homologs from multiple species, including mouse and zebrafish, and human FTO, ALKBH2 and ALKBH3. (b) Inhibition of ALKBH3 with gradient concentrations of **TD19** in PAGE-based assay.

Fig. S2

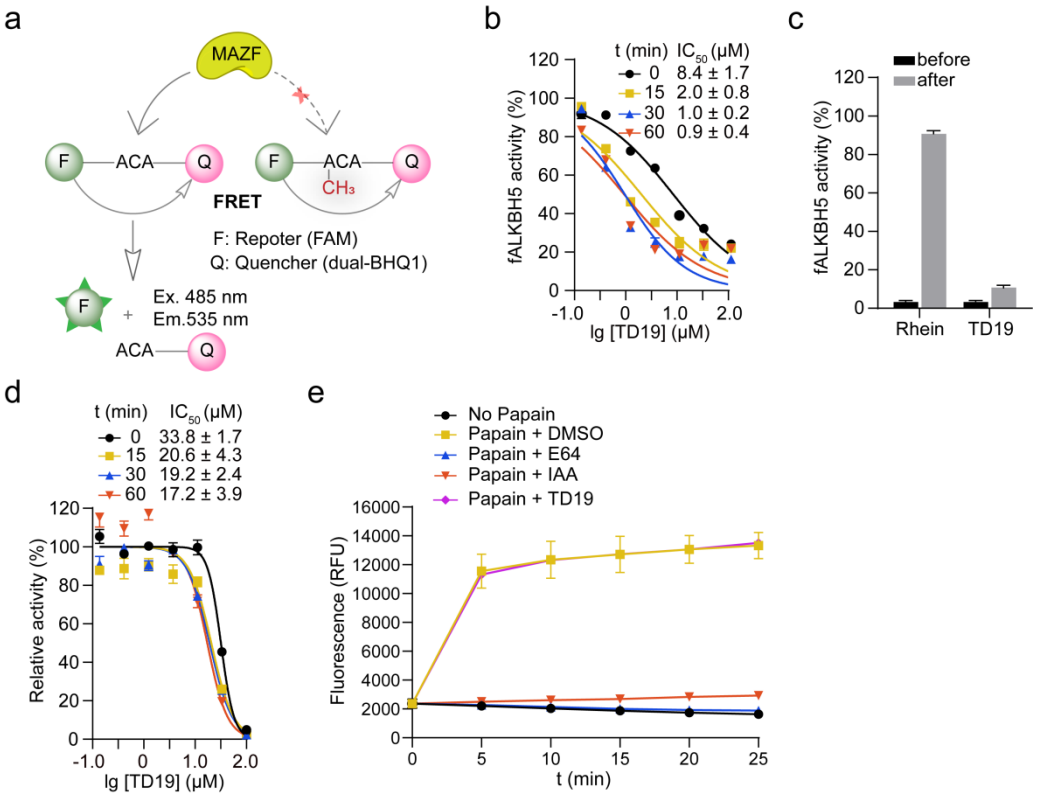


Fig. S2. TD19 is an irreversible inhibitor of ALKBH5. (a) Schematic representation of FRET-based m^{6}A detection assay.[6] MazF enzyme is used to specifically cleave single-stranded RNA at ACA sites, but not (m^{6}A)CA sites. RNA chimera probes containing a FRET pair (5'-FAM/3'-dual-BHQ1) are combined with MazF. When the RNA chimera probe is cleaved by MazF at the ACA site, the FRET signal is disrupted, resulting in an increase in fluorescence intensity of the reporter FAM (Ex. 485 nm, Em. 535 nm). The change in fluorescence signals is then monitored to indicate the demethylase activity. (b) Time-dependent irreversible inhibition of fALKBH5 by **TD19**, with IC_{50} values. (c) Effect of 5 μM **TD19** and 100 μM **rhein** on the demethylation by ALKBH5 in a buffer replacement assay. The control samples (treated with DMSO) were used as the 100% activity reference. (d) Inhibition of **rhein** on ALKBH5 after pre-incubation for varying durations (15 min, 30 min, 60 min). (e) Effects of **E64**, **IAA** and **TD19** on the cysteine protease papain.

Fig. S3

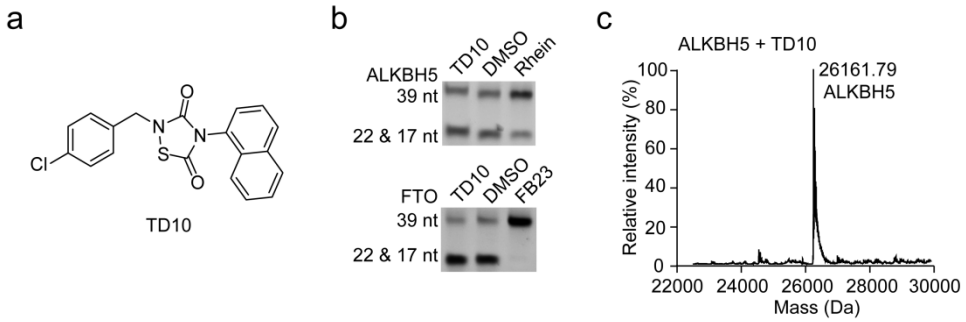


Fig. S3. TD10 does not exhibit inhibitory or modifying effects on ALKBH5**.** (a) Chemical structure of **TD10**. (b) The inhibitory effect of **TD10** on ALKBH5 and FTO at the concentration of 50 μM by PAGE-based assay. (c) Mass spectrometry detection of ALKBH5 incubated with **TD10** under the same condition as **TD19**.

Fig. S4

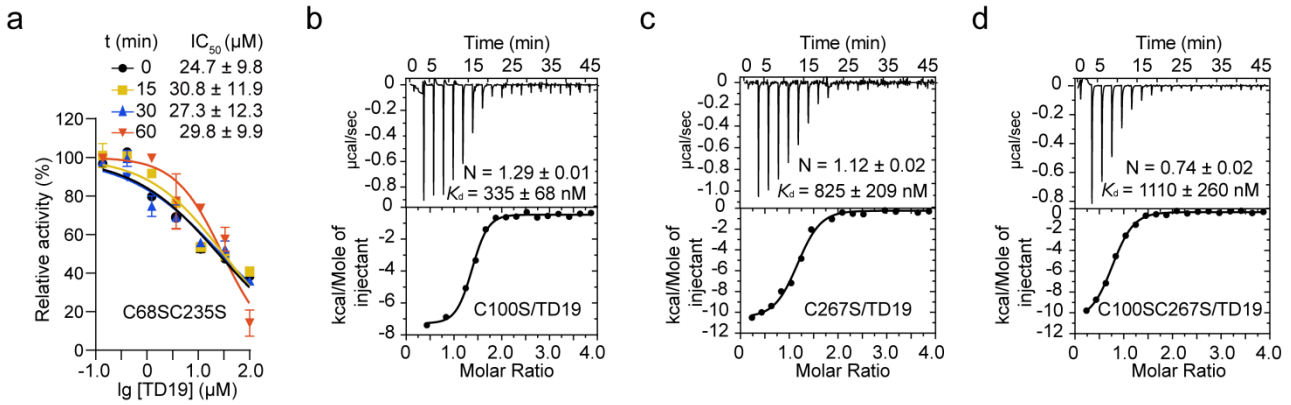


Fig. S4. Modification of **TD19** on C267 mainly contributed to the ALKBH5 enzymatic inhibition. (a) Time-dependent irreversible inhibition of **TD19** on fALKBH5 C68SC235S proteins. (b–d) Characterization of **TD19** binding to ALKBH5 C100S, C267S, and C100SC267S mutants by ITC titration, respectively. The dissociation constant (K_d) and stoichiometry factor (N) are indicated.

Fig. S5

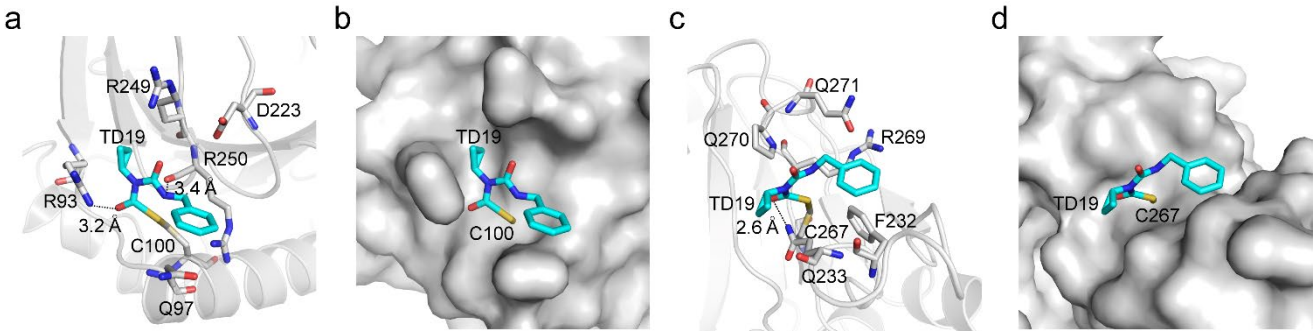


Fig. S5. Covalent docking models of **TD19** with ALKBH5. (a-b) Docking of **TD19** with C100 of ALKBH5. (c-d) Docking of **TD19** with C267 of ALKBH5. **TD19** is represented in cyan sticks, and the protein backbone is depicted in a grey cartoon and/or surface. Dark dotted lines indicate hydrogen bonding, and the distance in angstroms (Å) is labeled.

Fig. S6

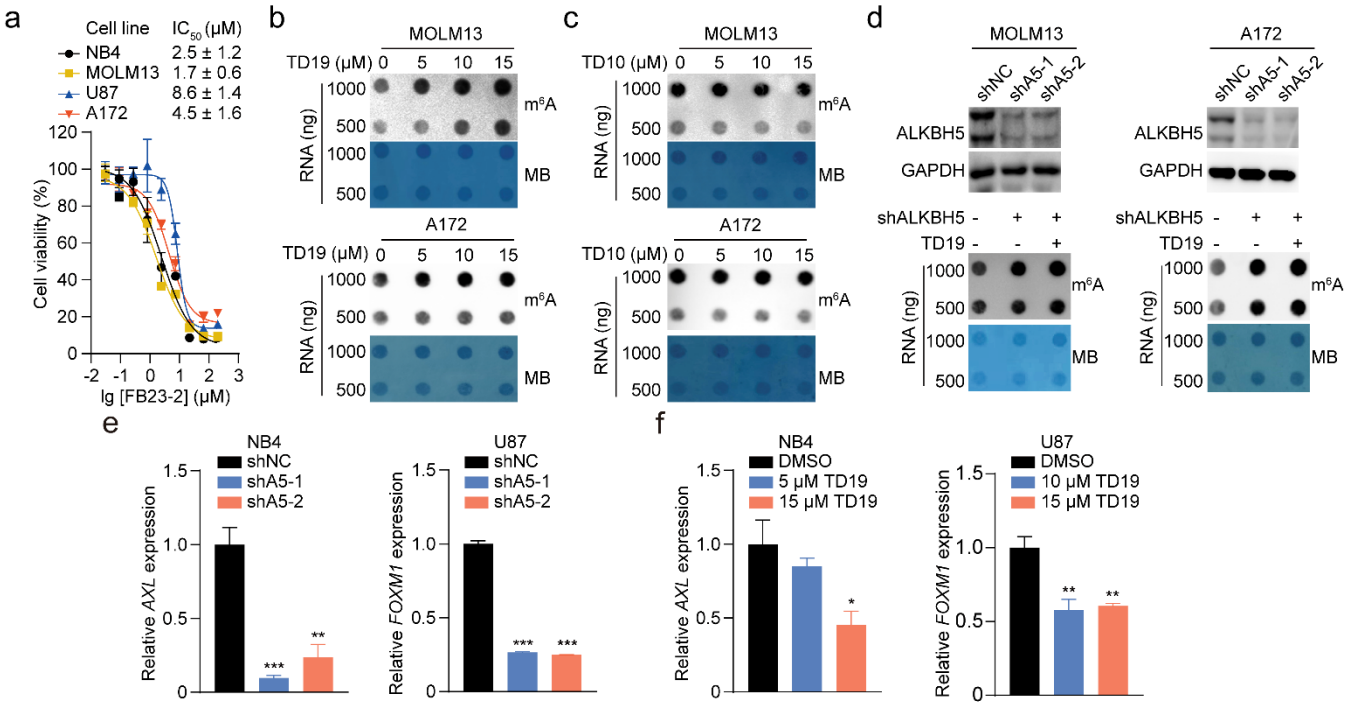
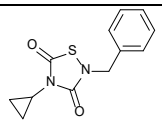
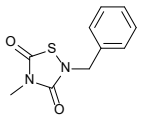
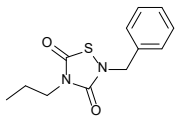
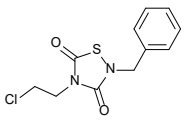
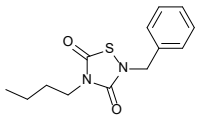
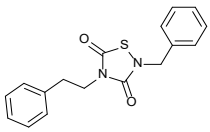
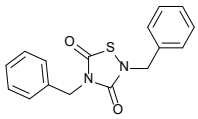
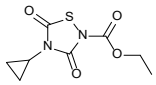
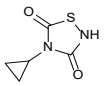


Fig. S6. TD19 targets ALKBH5 and regulates m^{6}A level and downstream gene expression at the cellular levels. (a) Effect of **FB23-2** on the viability of AML and GBM cells. The cells were treated with **FB23-2** for 72 h at the indicated concentrations before MTT/CCK8 measurement. (b) Determination of RNA m^{6}A abundance in MOLM13 and A172 cells following 72 h of **TD19** treatment using the dot blot assay. MB (methylene blue) was used as the loading control for RNA samples. (c) Determination of RNA m^{6}A abundance in MOLM13 and A172 cells following 72 h of **TD10** treatment using the dot blot assay. (d) Determination of ALKBH5 abundance in MOLM13 and A172 cells measured by western blot and effects of **TD19** and shALKBH5 on m^{6}A abundance in both cells, respectively. (e) Detection of *AXL* and *FOXM1* mRNA expression in ALKBH5 knockdown NB4 and U87 cells by RT-qPCR, respectively. (f) Effects of **TD19** treatment of 72 h on *AXL* and *FOXM1* mRNA expression in NB4 AML cells and U87 GBM cells by RT-qPCR, respectively. The results were obtained from three biological replicates. Statistical significance is denoted as *p < 0.05, **p < 0.01, and ***p < 0.001 using the unpaired Student's t-test. The error bars represent mean ± SD, n = 3.

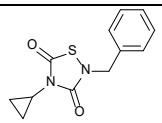
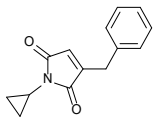
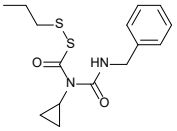
Supplemental Tables

Table S1. The inhibitory activities of **TD19** and its analogs (**S1** to **S8**) against ALKBH5 and FTO.

Comp.	Formula	Enzyme activity (μM)[a]	
		ALKBH5	FTO
TD19		1.5-3	> 100
S1		6-12	> 50
S2		12-25	> 50
S3		12-25	> 50
S4		12-25	> 50
S5		≈ 50	> 50
S6		≈ 12	> 50
S7		≈ 25	> 50
S8		> 50	ND

[a] Enzyme activity of test compounds were determined by a PAGE-based assay. ND = not detected.

Table S2. The inhibitory activities of **TD19** derivatives (**S9** and **S10**) against ALKBH5 and FTO.

Comp.	Formula	Enzyme activity (μM)[a]	
		ALKBH5	FTO
TD19		1.5-3	> 100
S9		> 50	> 50
S10		≈ 50	> 50

[a] Enzyme activity of test compounds were determined by a PAGE-based assay.

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