Supporting Information for:

Enhanced suppression of a protein-protein interaction in cells using small-molecule covalent inhibitors based on *N*-acyl-*N*-alkyl sulfonamide warhead

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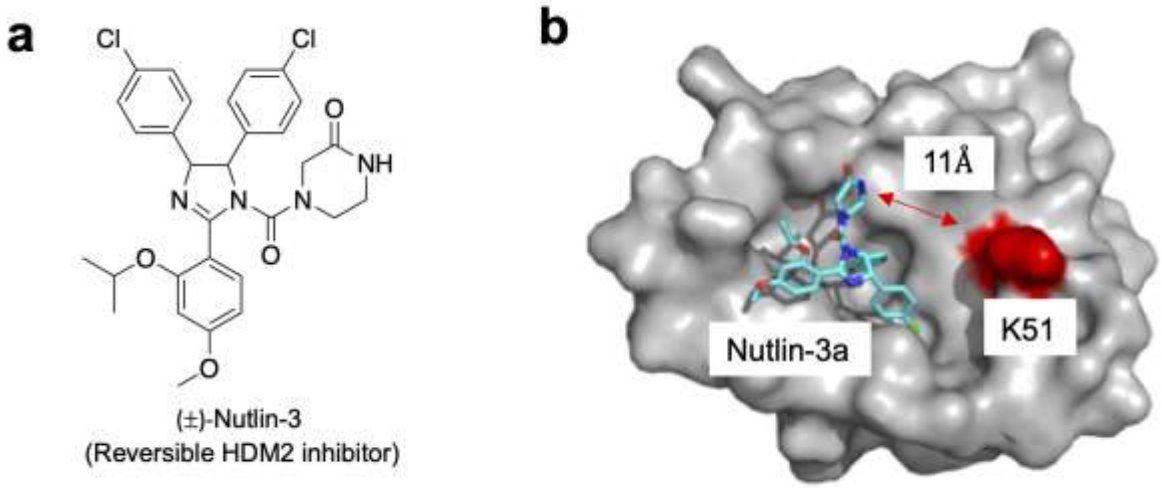


Figure S1 (a) Molecular structure of (±)-Nutlin-3. (b) Crystal structure of the N-terminal domain of HDM2 (17–108 a.a.)–Nutlin-3a complex (PDB ID; 4HG7). Lysine 51 (K51) is highlighted in red, and Nutlin-3a ligand is colored in cyan.

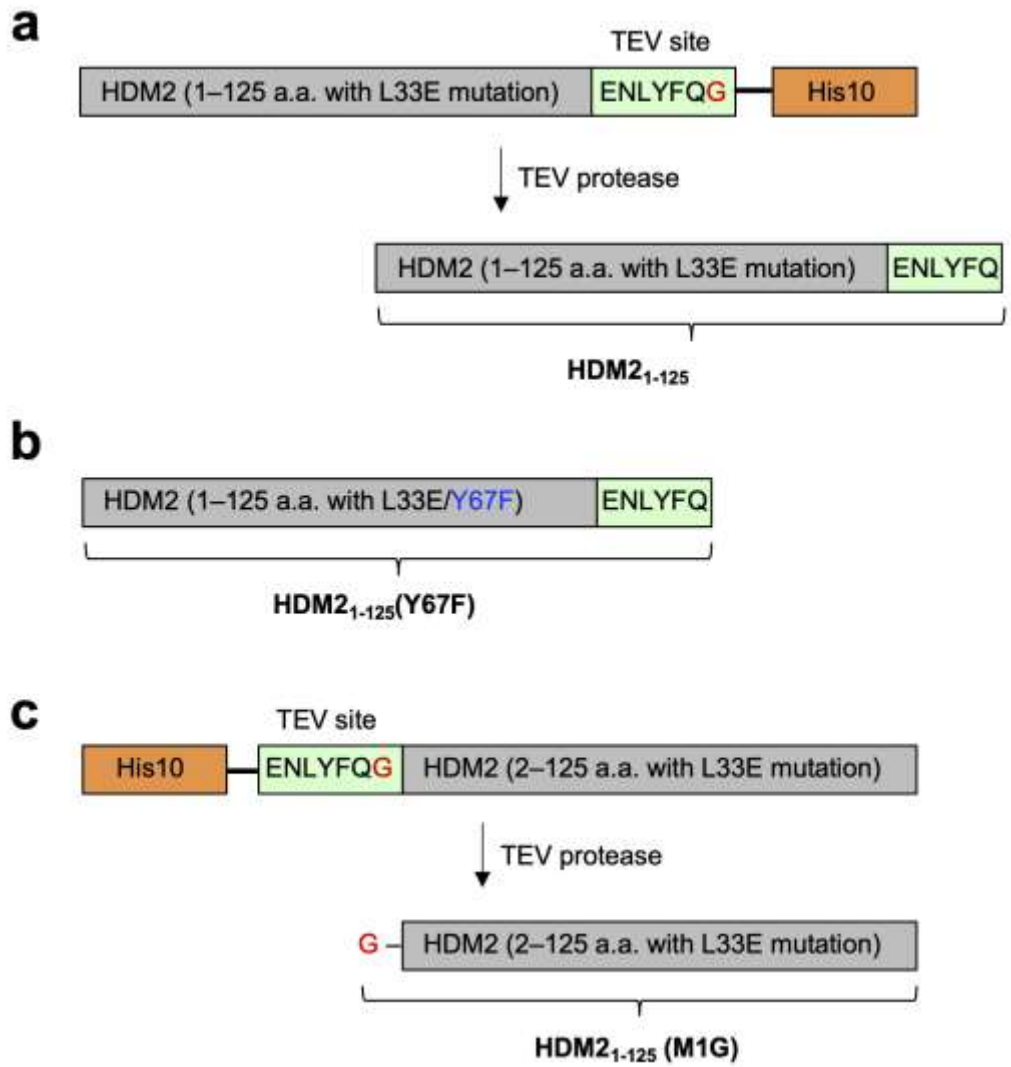


Figure S2 Primary structures of recombinant N-terminal domain of HDM2 used in this work. (a) N-terminal domain of HDM2 (1–125 a.a.) with a L33E mutation ($HDM2_{1-125}$) was obtained by bacterial expression as a fusion protein with a His10 tag and a TEV protease cleavage site at its C-terminus. (b) $HDM2_{1-125}$ (Y67F) to evaluate the modification of tyrosine 67. (c) $HDM2_{1-125}$ (M1G). $HDM2_{1-125}$ (M1G), a more stable variant than $HDM2_{1-125}$, was used in the SEC-based studies (Figure 3d).

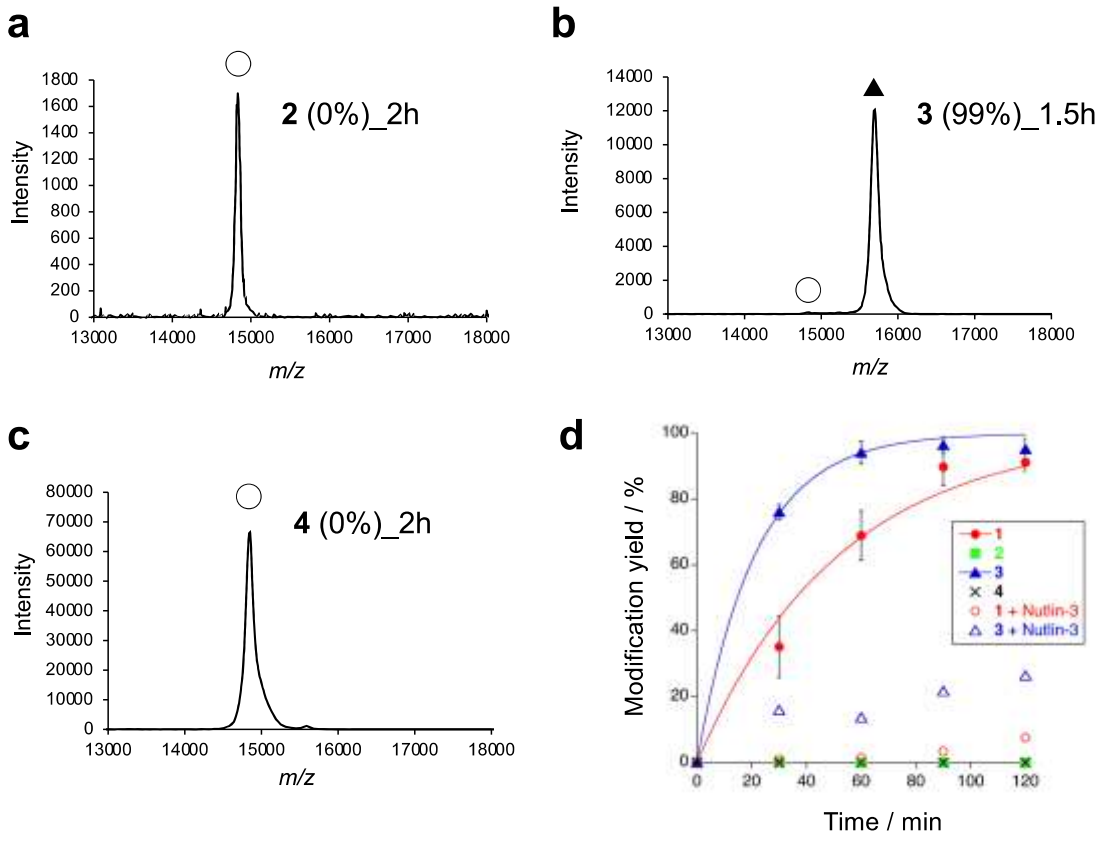


Figure S3 Time profile for the covalent modification of $HDM2_{1–125}$ with covalent inhibitors. (a–c) MALDI-TOF MS analysis of covalent modification of recombinant $HDM2_{1–125}$ with **2**–**4**. Reaction conditions: 1 μM $HDM2_{1–125}$, 3 μM covalent inhibitor, 30 μM (±)-Nutlin-3 (as a competitor), PBS buffer, pH 7.4, 37 °C, 2 h. ○, native $HDM2_{1–125}$ (M_w : 14 955); ▲, covalent adduct of the $HDM2_{1–125}$ with **3** (M_w : 15 822). (d) Time courses of the reaction yields for **1**–**4**, **1**+(±)-Nutlin-3 and **3**+(±)-Nutlin-3. Error bars represent standard deviation of the mean, n = 3.

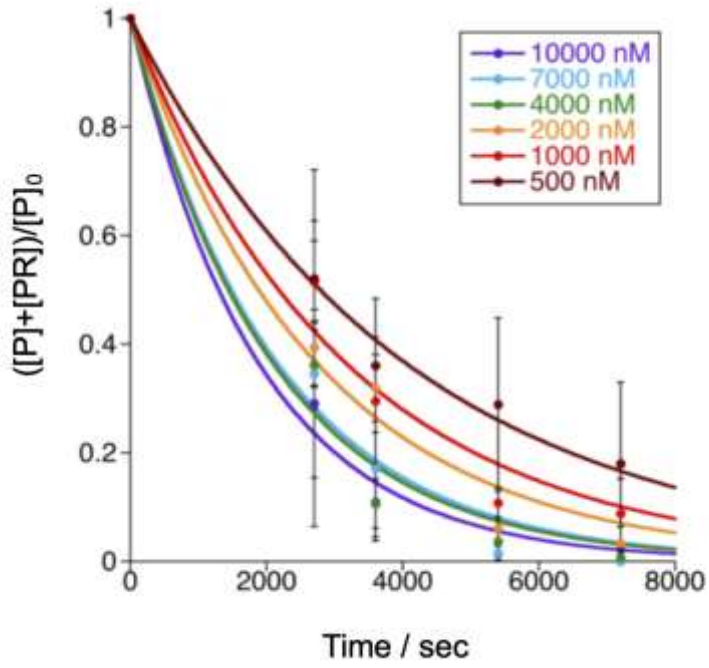


Figure S4 Time courses of the depletion of native (non-labeled) $HDM2_{1\text{-}125}$ ([P]) during the labeling reaction with compound **1** ([R]) (n=3). A purified $HDM2_{1\text{-}125}$ (300 nM) was incubated with **1** (500–10000 nM) in PBS buffer (pH 7.4) at 37 °C. The pseudo-first order reaction rates (k_{app}) were obtained by fitting the data to equation (2) (see Method section). The dependence of k_{app} on the concentration of reagent **1** is shown in Figure 3b. Kinetic parameters for covalent inhibition (k_{inact} and K_d) were obtained by fitting the data of Figure 3b to equation (3). Error bars represent standard deviation of the mean, n = 3.

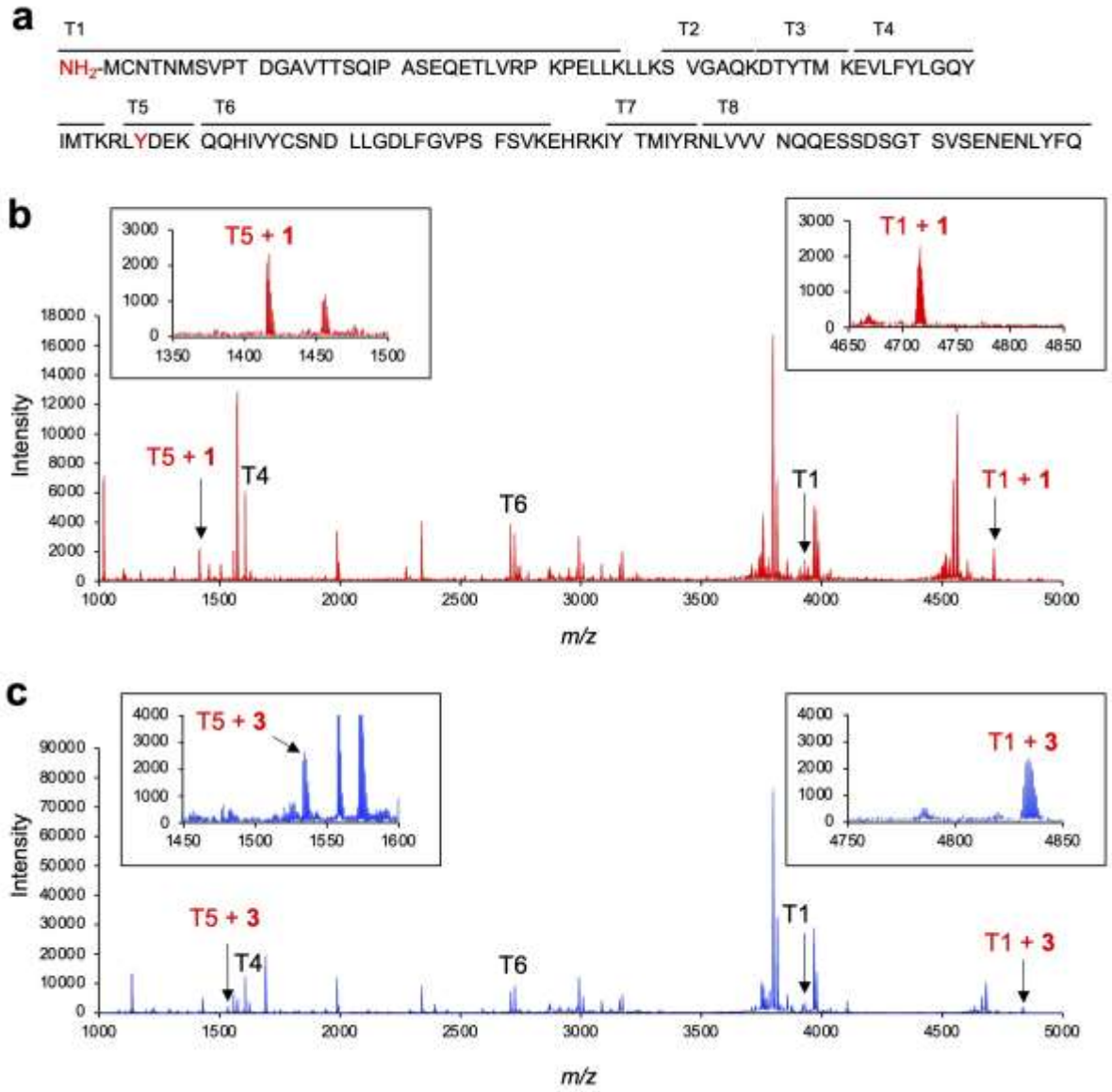


Figure S5 Mass spectral analysis of modification sites of $HDM2_{1-125}$ with covalent inhibitors. (a) The primary sequence of $HDM2_{1-125}$ and predictable fragments generated by tryptic digestion (T1–T8). The modification sites by **1** and **3** are shown in red (N-terminal amino group and Tyr67). (b) MALDI-TOF MS analysis of the digested fragments of **1**-modified $HDM2_{1-125}$. The peaks marked with the character (e.g. T1) correspond to the identified peptide fragments. T1 (carbamidomethylated on Cys): calcd. for $[M+Na]^+$ = 3964.9, obsd. 3965.2, T4: calcd. for $[M+H]^+$ = 1604.8, obsd. 1605.5, T6 (carbamidomethylated on Cys): calcd. for $[M+H]^+$ = 2723.3, obsd. 2724.4, T1+**1** (modified with compound **1**, carbamidomethylated on Cys): calcd. for $[M+Na]^+$ = 4712.1, obsd. 4712.4, T5+**1**: calcd. for $[M+H]^+$ = 1414.3, obsd. 1415.2. (c) MALDI-TOF MS analysis of the digested fragments of

3-modified $HDM2_{1\text{-}125}$. T1+**3** (modified with compound **3**, carbamidomethylated on Cys): calcd. for $[M+Na]^+$ = 4830.2, obsd. 4830.7, T5+**3**: calcd. for $[M+H]^+$ = 1532.6, obsd. 1533.4.

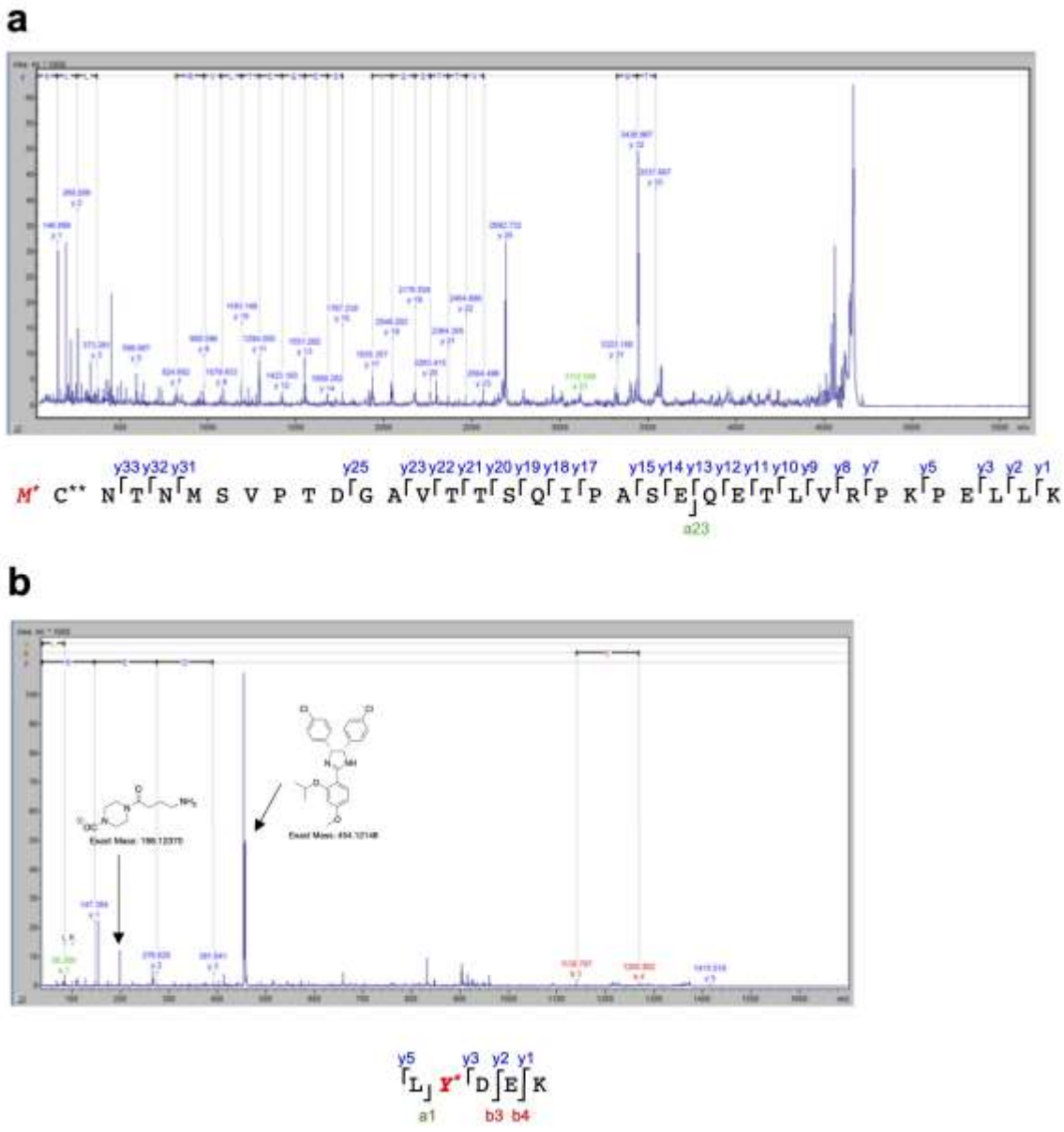


Figure S6 MALDI-TOF/TOF MS analysis of peptides modified with **1**. Fragment mass peaks derived from (a) T1+**1** (parent MS: 4712.4 Da) and (b) T5+**1** (parent MS: 1415.2 Da). *, the modification site of **1**, **, carbamidomethylated cysteine. In (a), a y33 ion was detected, indicating that the modification site should be Met1 (N-terminal amine), Cys2 or Asn3. There is no possibility that the Cys2 is modified with **1** because it is carbamidomethylated by iodoacetamide before tryptic digestion. The side chain of Asn is a non-nucleophilic amide group, which cannot react with the NASA group. Therefore, the N-terminal amino group was identified as one of the modification sites of **1**. In (b), b3 ion, corresponding to **1**-modified Leu-Tyr-Asp, was observed. The detection of y3 ions indicates that no modification to Asp

has occurred. Since leucine does not have any reactive group, the tyrosine should be modified with **1**. This result is also supported by the facts that: (i) two fragments bearing the Nutlin moiety were observed, indicating the parent MS (1415.2 Da) is derived from the peptide modified with **1**; (ii) y3 (DEK), y2 (EK) and y1 ion (K) was detected, suggesting that the DEK sequence is not modified with **1**. Thus, Tyr67 was identified as another modification site of **1**.

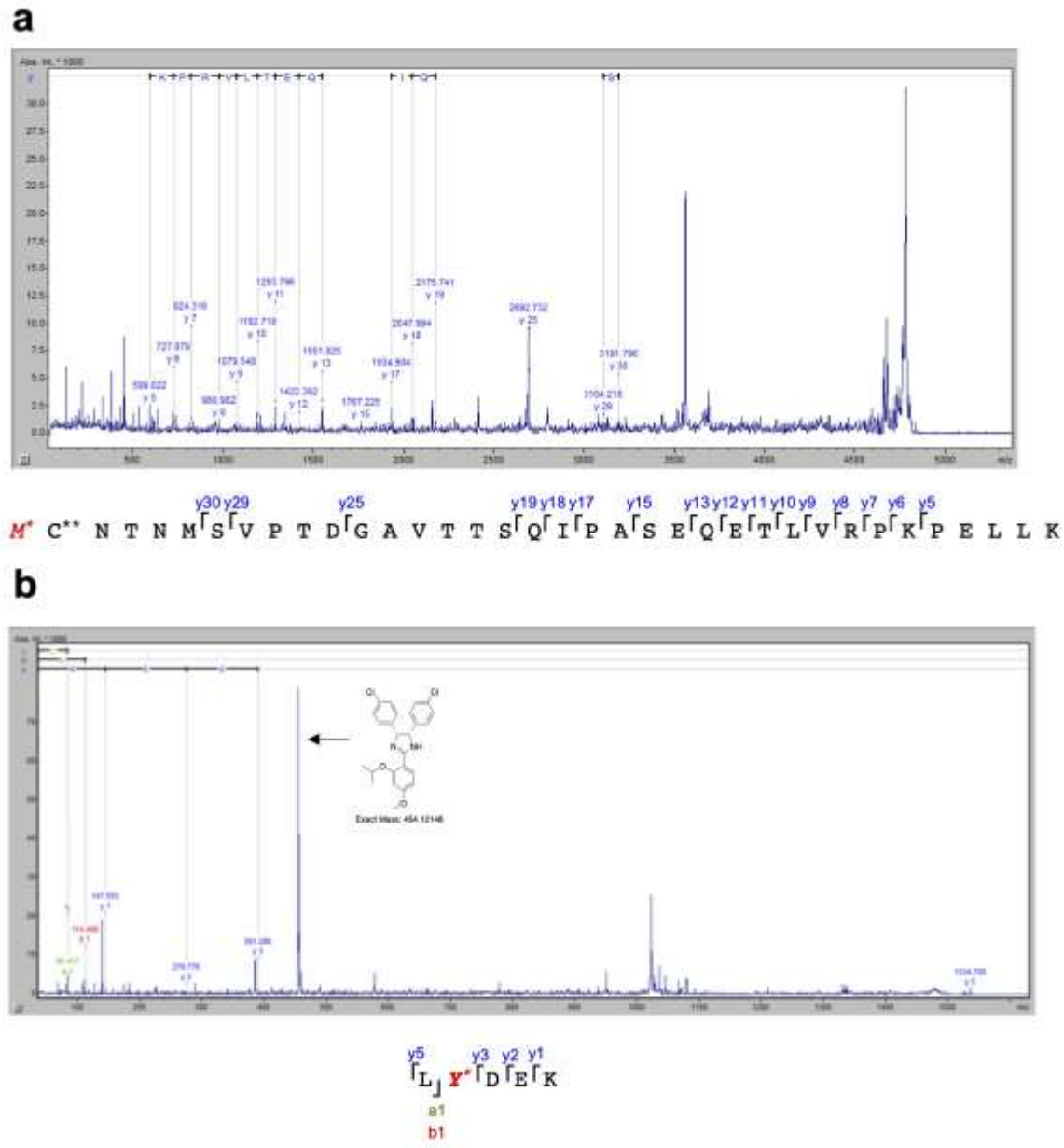


Figure S7 MALDI-TOF/TOF MS analysis of peptides modified with **3**. Fragment mass peaks derived from (a) T1+**3** (parent MS: 4830.7 Da) and (b) T5+**3** (parent MS: 1533.4 Da). *, the modification site of **3**, **, carbamidomethylated cysteine. In (a), a y30 ion was detected, indicating that the modification site should be in the range of Met1 (N-terminal amine) to Met6. As mentioned above, there is no possibility that Cys2, Asn3, and Asn5 are modified with **3**. The side chain of Met is a non-nucleophilic thioether group, which cannot be acylated with the NASA group. Although this MSMS data does not rule out the modification to Thr4, MALDI-ISD in Figure S8 clearly demonstrates the N-terminal modification of **3**. In (b), the

detection of y3 ions indicates that no modification to DEK has occurred. Since leucine does not have any reactive group, the tyrosine should be modified with **3**.

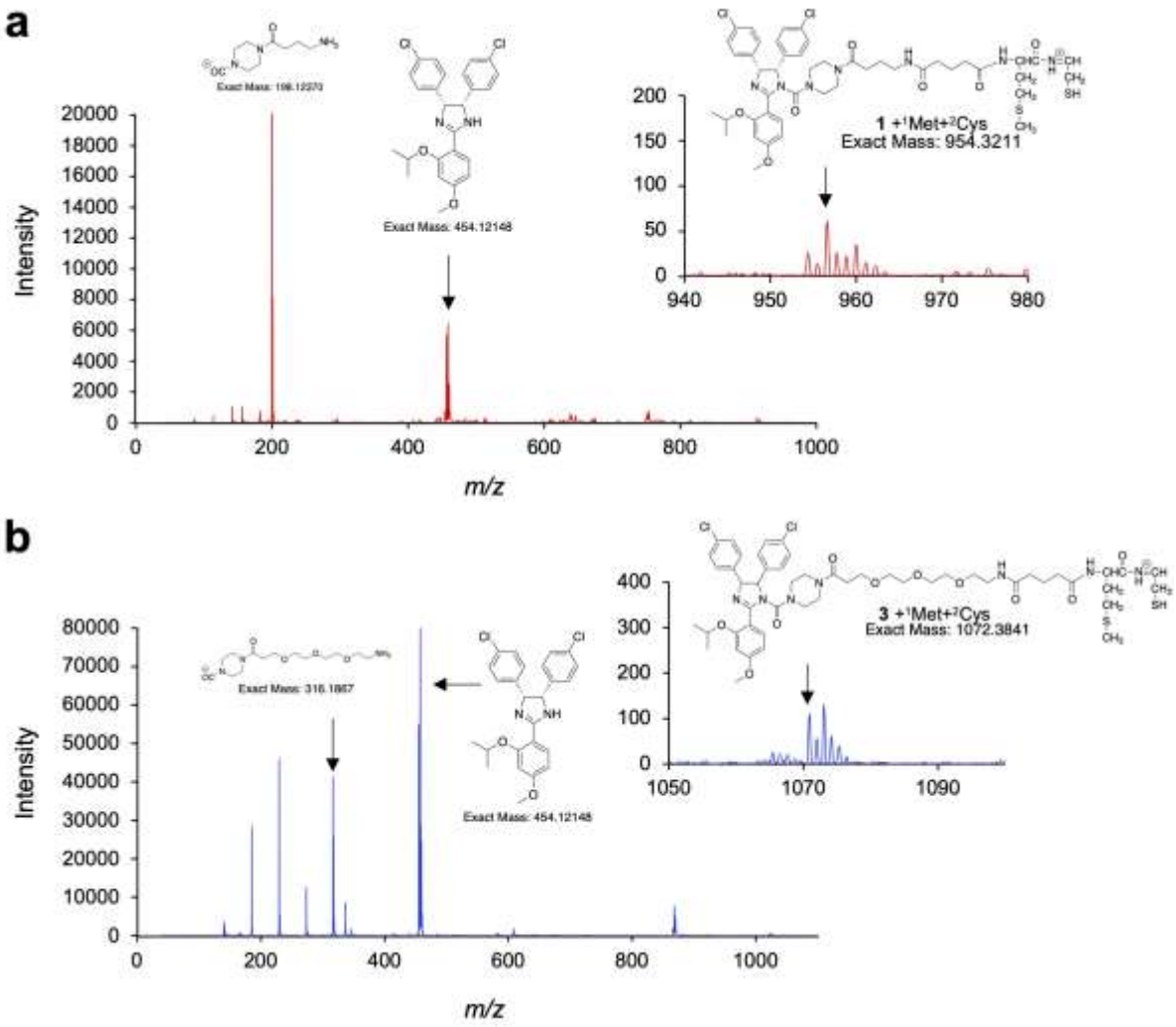


Figure S8 MALDI-in-source decay (ISD) measurements of the modified $HDM2_{1\text{-}125}$ for N-terminal structure analysis. MALDI-ISD with the subsequent TOF/TOF-MS analyses of (a) **1**+^{1}Met+^{2}Cys (parent MS: 954.5 Da) and (b) **3**+^{1}Met+^{2}Cys (parent MS: 1072.9 Da). In both cases, two fragments peaks of NASA-based inhibitors (Nutlin scaffold (obsd. 455.6 Da) and the spacer moieties (obsd. 199.8 and 316.4)) were observed, proving that these parent mass ions are derived from molecules modified with **1** or **3**. Taken together with MSMS data in Figure S6 and S7, the modification to the N-terminal amino group was clearly identified.

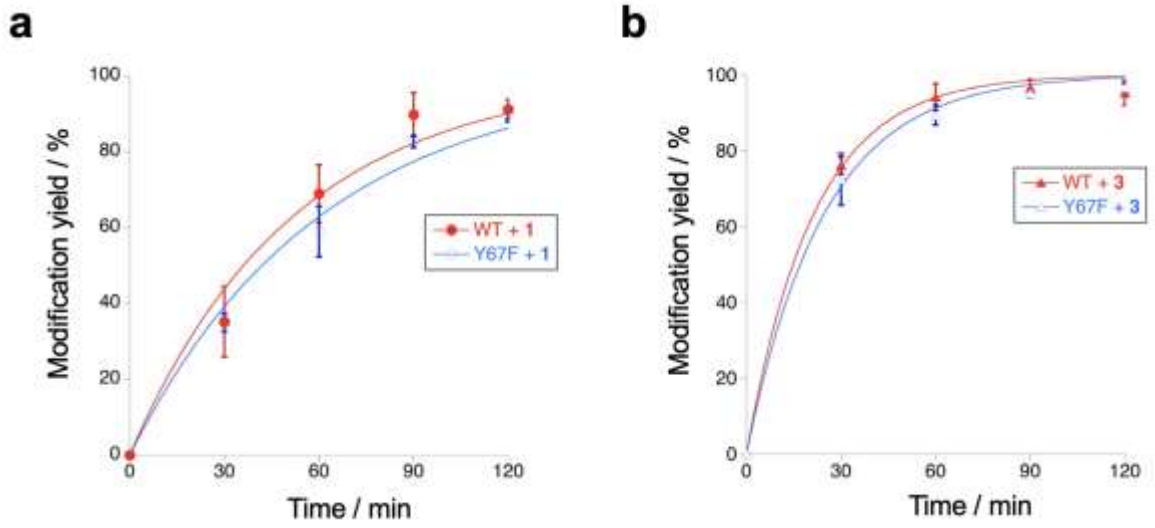


Figure S9 Covalent modification of $HDM2_{1\text{-}125}$ (WT) and $HDM2_{1\text{-}125}$ (Y67F) with (a) **1** and (b) **3**. Error bars represent standard deviation of the mean, n = 3.

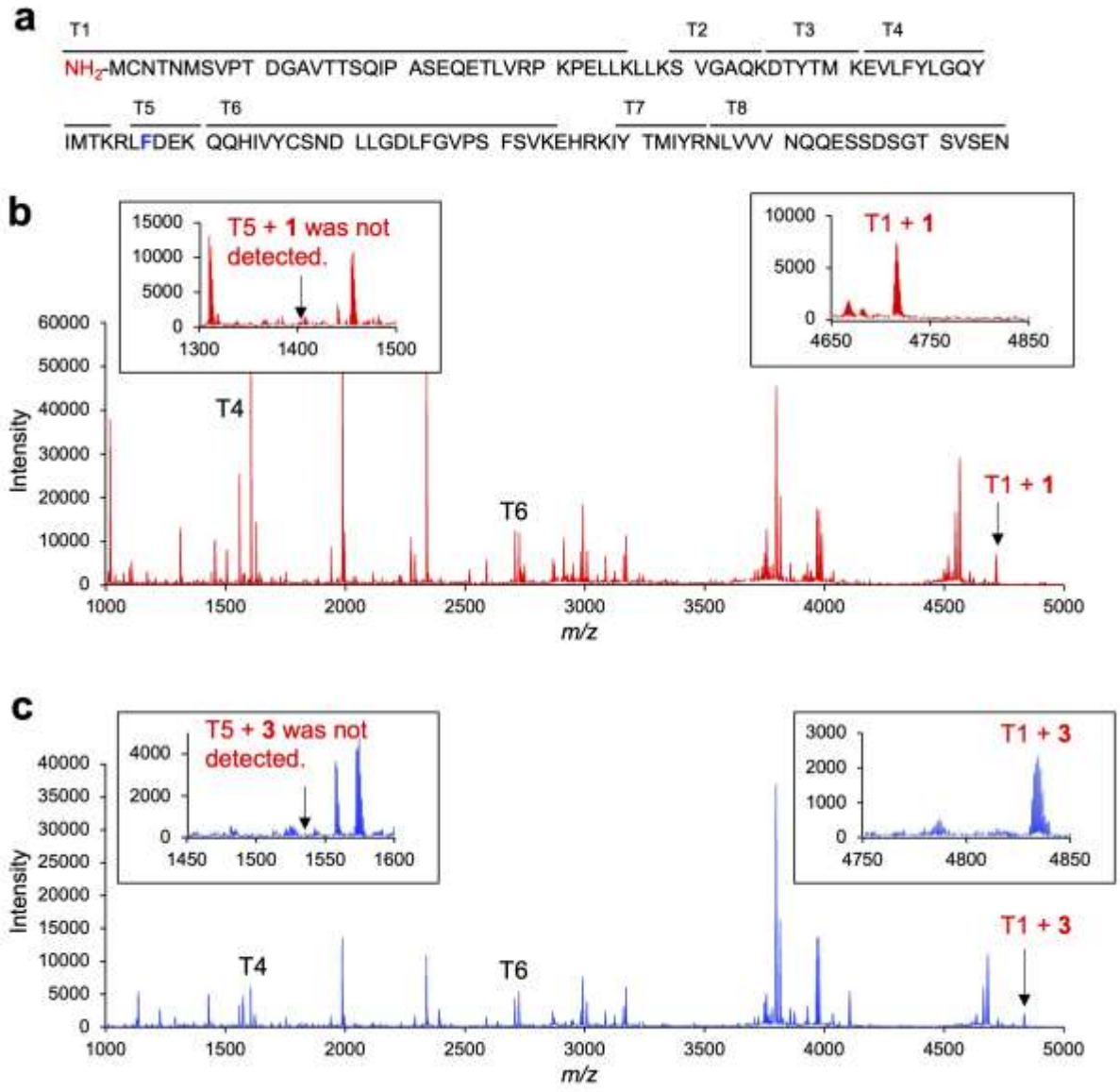


Figure S10 Mass spectral analysis of modification sites of $HDM2_{1\text{-}125}$ (Y67F) with covalent inhibitors. (a) The primary sequence of $HDM2_{1\text{-}125}$ (Y67F) and predictable fragments generated by tryptic digestion (T1–T8). The modification sites by **1** and **3** are shown in red (N-terminal amino group). (b) MALDI-TOF MS analysis of the digested fragments of **1**-modified $HDM2_{1\text{-}125}$ (Y67F). The peaks marked with the character (e.g. T1) correspond to the identified peptide fragments. T4: calcd. for $[M+H]^+$ = 1604.8, obsd. 1605.6, T6 (carbamidomethylated on Cys): calcd. for $[M+H]^+$ = 2723.3, obsd. 2724.4, T1+**1** (modified with compound **1**, carbamidomethylated on Cys): calcd. for $[M+Na]^+$ = 4712.1, obsd. 4712.5. A peak corresponding to T5+**1** was not observed. (c) MALDI-TOF MS analysis of the digested fragments of **3**-modified $HDM2_{1\text{-}125}$ (Y67F). T1+**3** (modified with compound **3**,

carbamidomethylated on Cys): calcd. for $[M+Na]^+$ = 4830.2, obsd. 4830.6. A peak corresponding to T5+**3** was not observed.

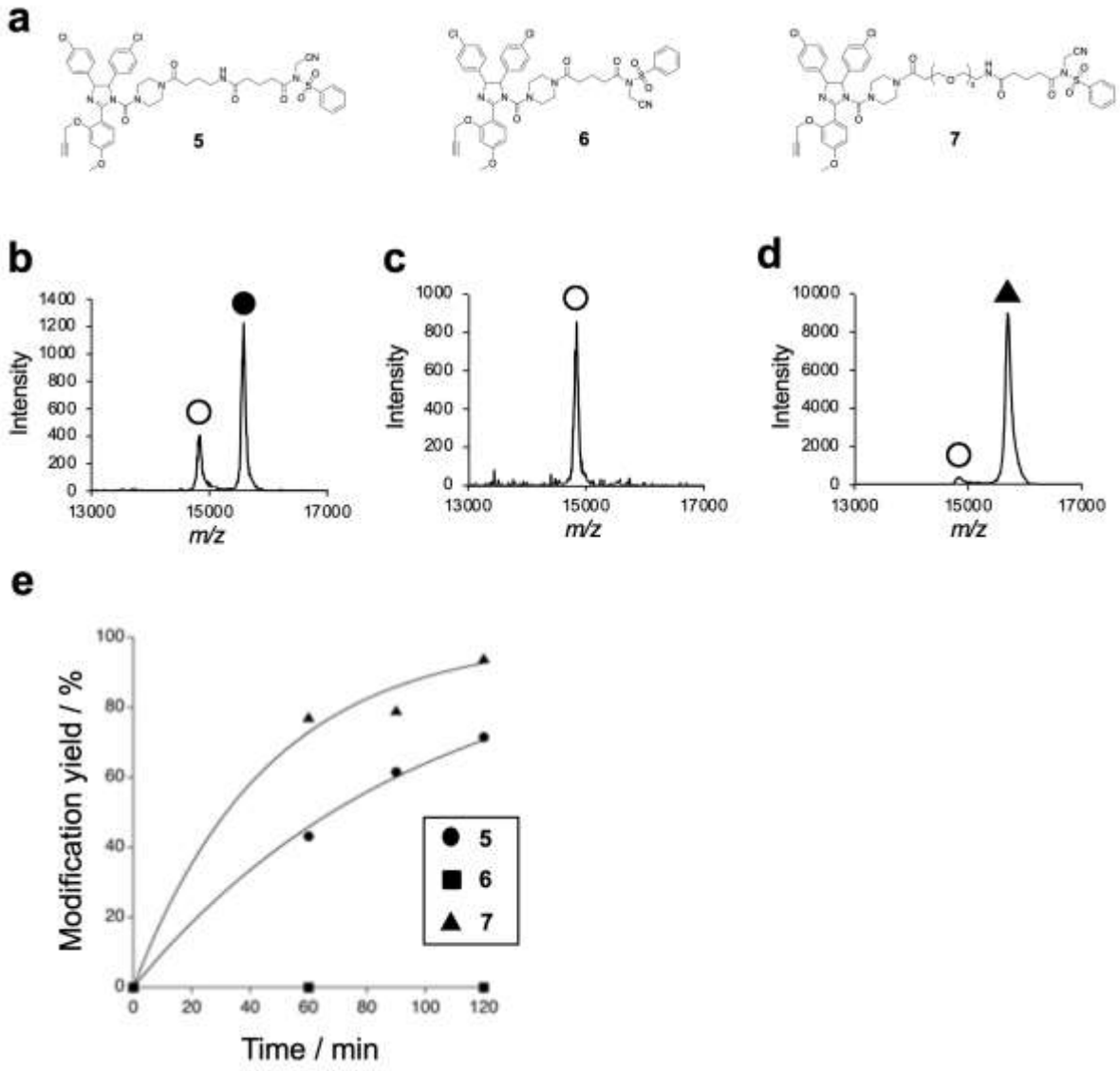


Figure S11 Reaction profile of the covalent modification of HDM2$_{1\text{-}125}$ with alkyne-appended probes **5**–**7**. (a) Molecular structures of probe **5**–**7**. (b–d) MALDI-TOF MS analysis of covalent modification of recombinant HDM2$_{1–125}$ with (b) **5**, (c) **6**, and (d) **7**. Reaction conditions: 1 μM HDM2$_{1–125}$, 3 μM probe, PBS buffer, pH 7.4, 37 °C, 2 h. ○, native HDM2$_{1–125}$ (M_w : 14 955); ●, covalent adduct of the HDM2$_{1–125}$ with **5** (M_w : 15 699); ▲, covalent adduct of the HDM2$_{1–125}$ with **7** (M_w : 15 817). (b) Time courses of the reaction yields for **5**–**7**.

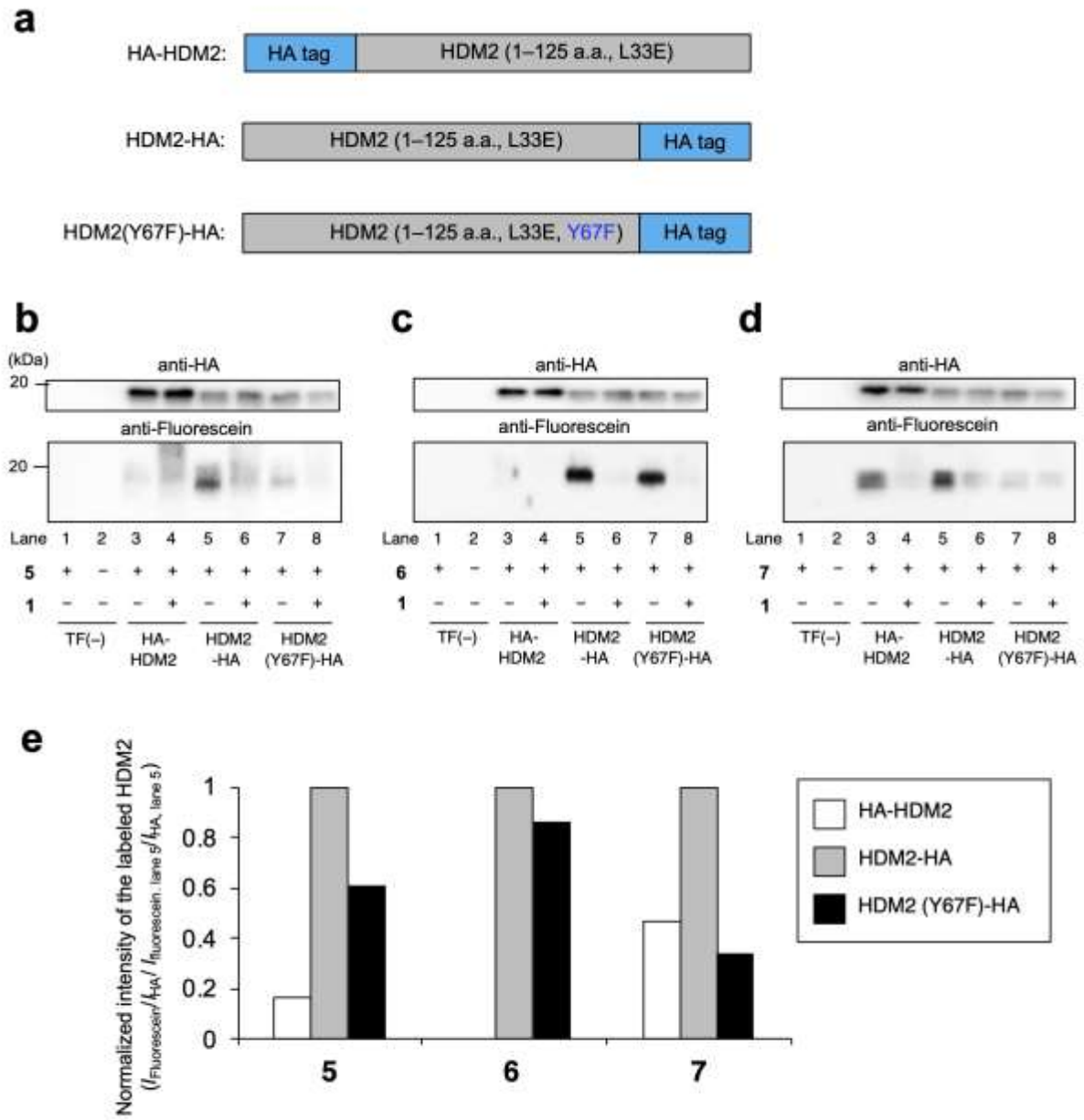


Figure S12 Cell-based evaluation of modification sites in HDM2 N-terminal domain with probes **5**–**7**. (a) Primary structures of N-terminal domain of HDM2 for this assay. 'HA-HDM2' is designed to mask the original N-terminus of $HDM2_{1\text{-}125}$ with an HA tag, which abrogates the labeling of N-terminal amino group by the probe. 'HDM2-HA' is a construct in which an HA tag is fused with $HDM2_{1\text{-}125}$ at the C-terminus. 'HDM2 (Y67F)-HA' is a variant of HDM2-HA in which Tyr67 is mutated to phenylalanine, prohibiting the modification on the residue. (b–d) HDM2 labeling in HEK293T cells with (b) **5**, (c) **6** and (d) **7**. TF, transfection. (e) Normalized band intensities of the labeled proteins (calculated with lane 3, 5, 7). The cells

expressing each variant of HDM2 N-terminal domain were treated with probe (0.3 μM) in the absence or presence of **1** (10 μM) for 1 h at 37 °C in medium (pH 7.4). After washing, the cells were lysed, subjected to click conjugation with fluorescein, and analyzed by western blotting using anti-HA and anti-fluorescein antibodies. In (b) and (d), the labeling bands of HA-HDM2 (lane 3) and HDM2 (Y67F)-HA (lane 7) were detected but substantially suppressed compared with that of HDM2-HA (lane 5), which indicates that both the N-terminal amino group and tyrosine 67 are involved in the reaction with **5** (an analog of **1**) and **7** (an analog of **3**) under live cell conditions. In (c), unlike the case of *in vitro* reaction (Figure S3 and S11), the short linker type compound **6** (an analog of **2**) can label HDM2-HA (lane 5) and HDM2 (Y67F)-HA (lane 7) with almost equal efficiency but did not react with HA-HDM2 at all (lane 3). This result reveals that the modification site with **6** is exclusively the N-terminal amino group of HDM2 under a live cell condition. This also suggests that the spatial configuration and/or dynamics of the N-terminus is significantly different between purified HDM2 and intracellular HDM2, presumably because the flexible N-terminal loop structure would vary depending on post-translational modifications, interactions with other proteins and/or molecular crowding environments.[S1]

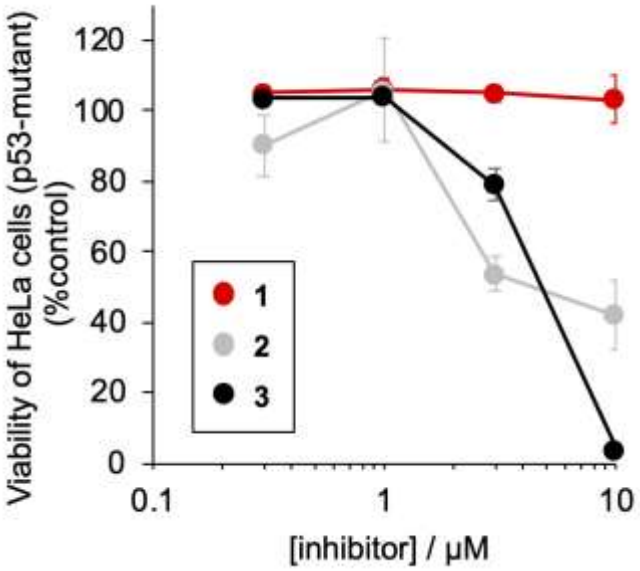


Figure S13 The effect of covalent inhibitors **1**–**3** on the growth and viability of HeLa cells bearing inactive mutant p53. Error bars represent standard error of mean, n = 4. Compounds **2** and **3** showed cytotoxic activity for HeLa cells at a concentration of 1–10 μM, whereas **1** did not. This data clarified that **2** and **3** show p53-independent cytotoxicity.

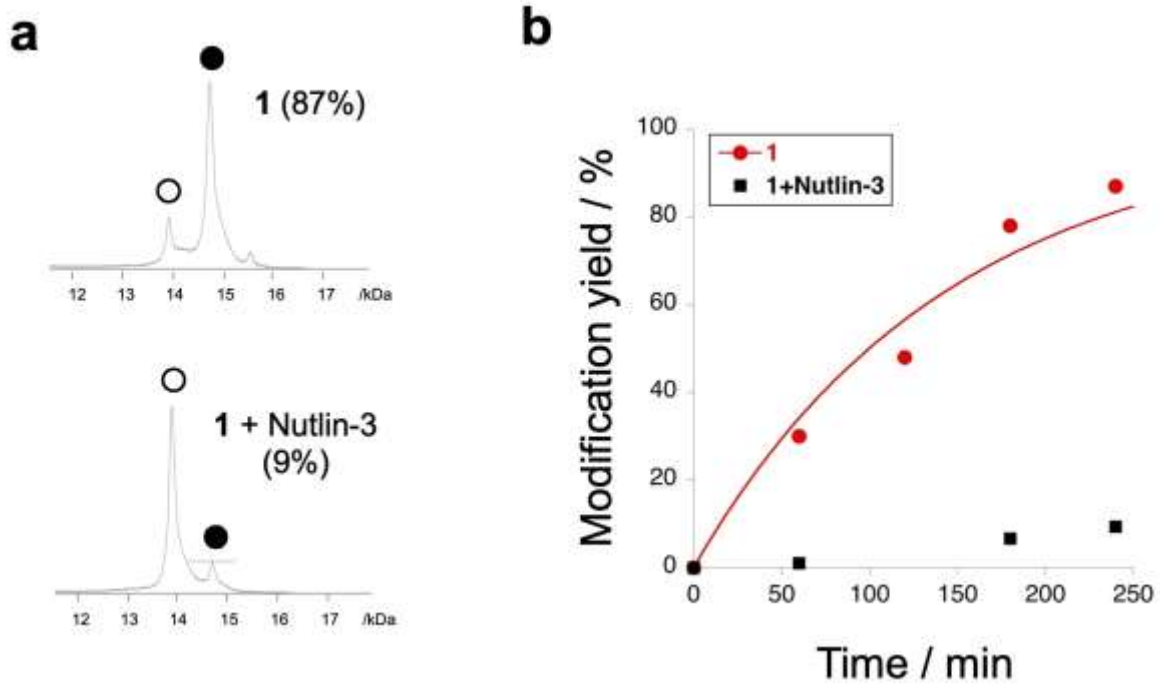


Figure S14 Time profile for the covalent modification of $HDM2_{1–125}$ (M1G) with **1**. (a) MALDI-TOF MS analysis of covalent modification of recombinant $HDM2_{1–125}$ (M1G) with **1**. Reaction conditions: 1 μM $HDM2_{1–125}$ (M1G), 3 μM **1**, 30 μM (±)-Nutlin-3 (as a competitor), PBS buffer, pH 7.4, 37 °C, 2 h. ○, non-labeled $HDM2_{1–125}$ (M1G) (M_w : 14 078); ●, covalent adduct of the $HDM2_{1–125}$ (M1G) with **1** (M_w : 14 826). (b) Time courses of the reaction yields for **1** and **1**+(±)-Nutlin.

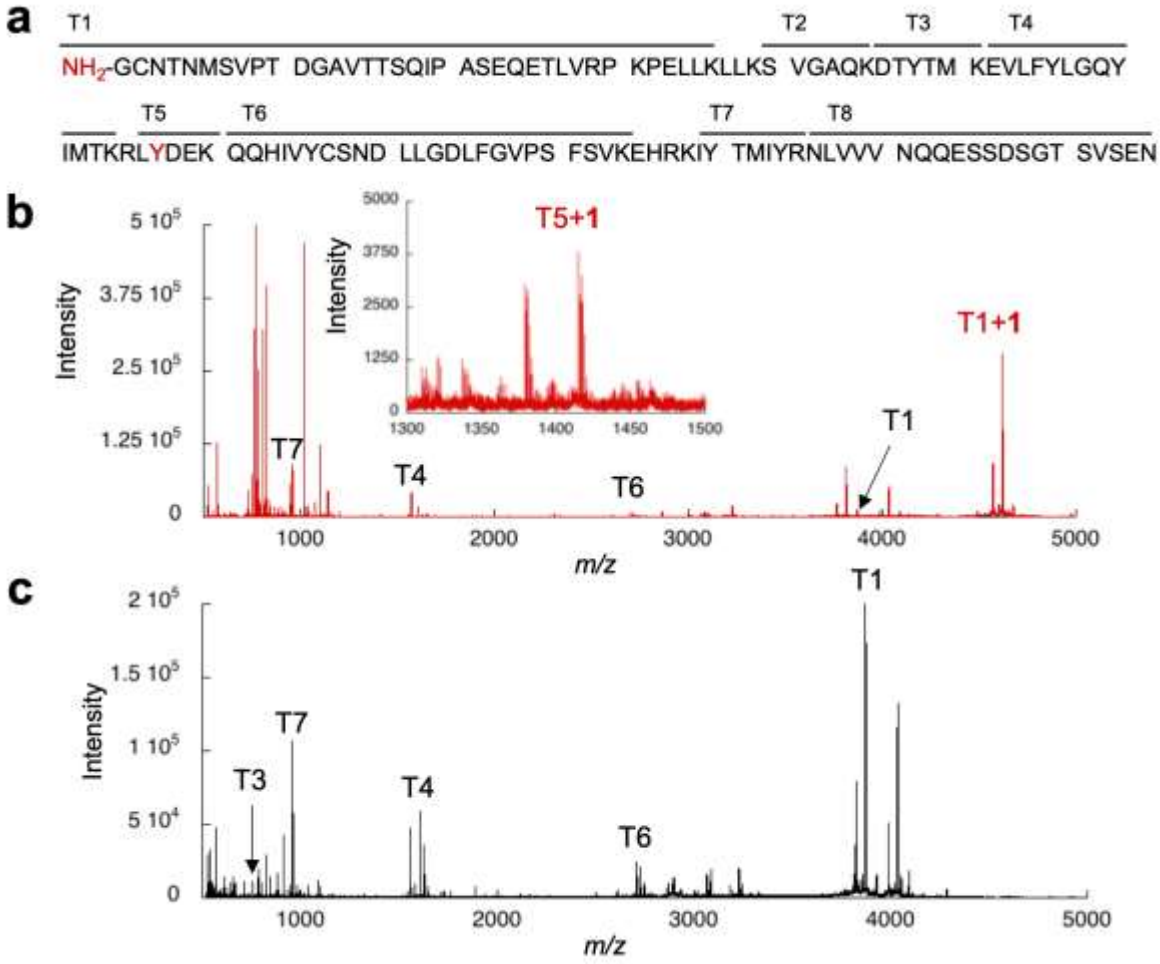


Figure S15 Mass spectral analysis of modification sites of $HDM2_{1–125}$ (M1G) with compound **1**. (a) The primary sequence of $HDM2_{1–125}$ (M1G) and predictable fragments generated by tryptic digestion (T1–T8). The modification sites by **1** are shown in red (N-terminal amino group and Tyr67). (b, c) MALDI-TOF MS analysis of the digested fragments of (b) modified and (c) unmodified $HDM2_{1–125}$ (M1G). The peaks marked with the character (e.g. T1) correspond to the identified peptide fragments. T1 (carbamidomethylated on Cys): calcd. for $[M+H]^+$ = 3868.9, obsd. 3869.2, T3: calcd. for $[M+H]^+$ = 758.3, obsd. 758.7, T4: calcd. for $[M+H]^+$ = 1604.8, obsd. 1605.4, T6 (carbamidomethylated on Cys): calcd. for $[M+H]^+$ = 2723.3, obsd. 2724.2, T7: calcd. for $[M+H]^+$ = 959.5, obsd. 959.9, T1+**1** (modified with **1**, carbamidomethylated on Cys): calcd. for $[M+H]^+$ = 4615.9, obsd. 4616.1, T5+**1** (modified with (±)-Nutlin-3): calcd. for $[M+H]^+$ = 1414.3, obsd. 1415.0.

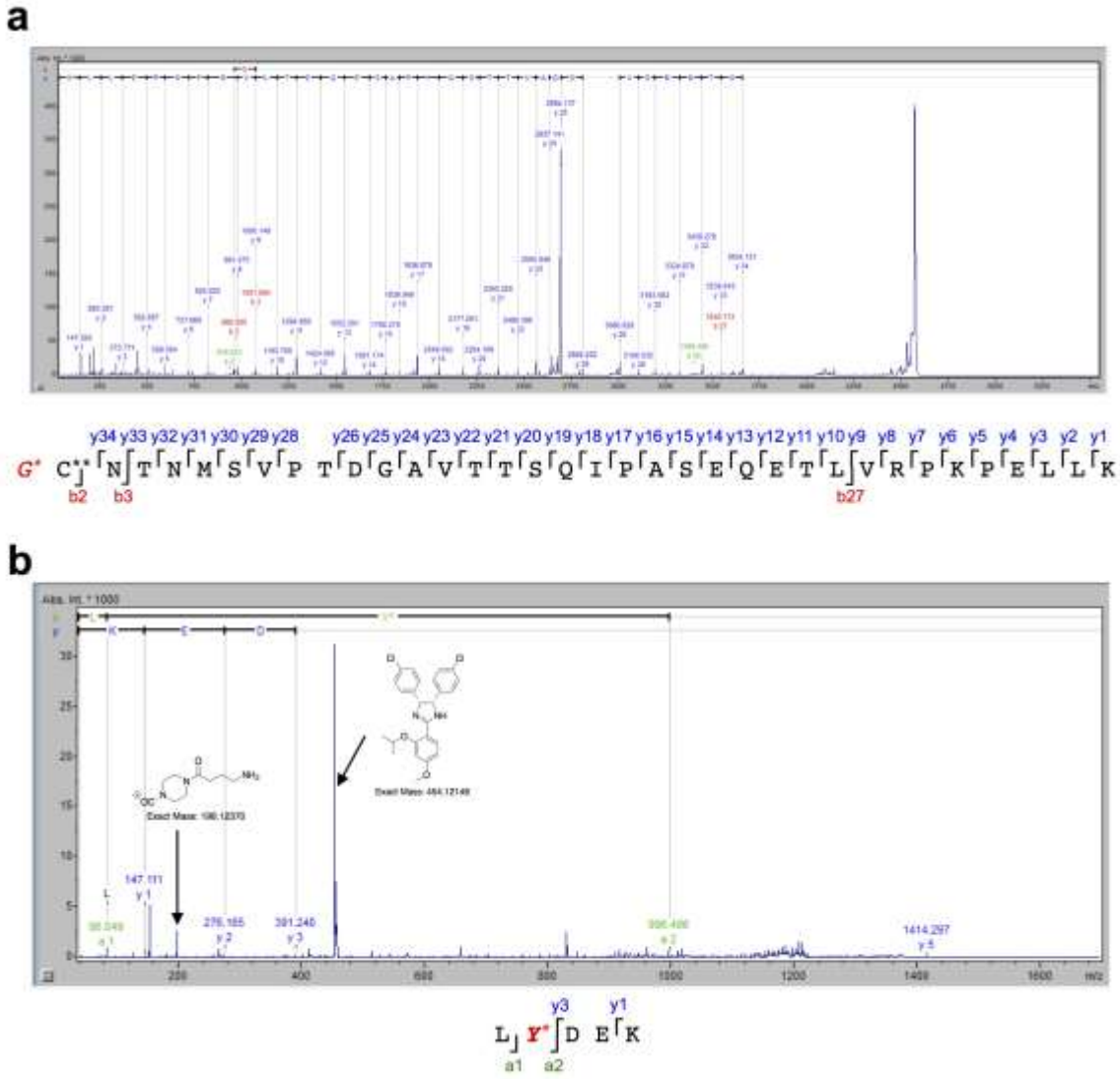
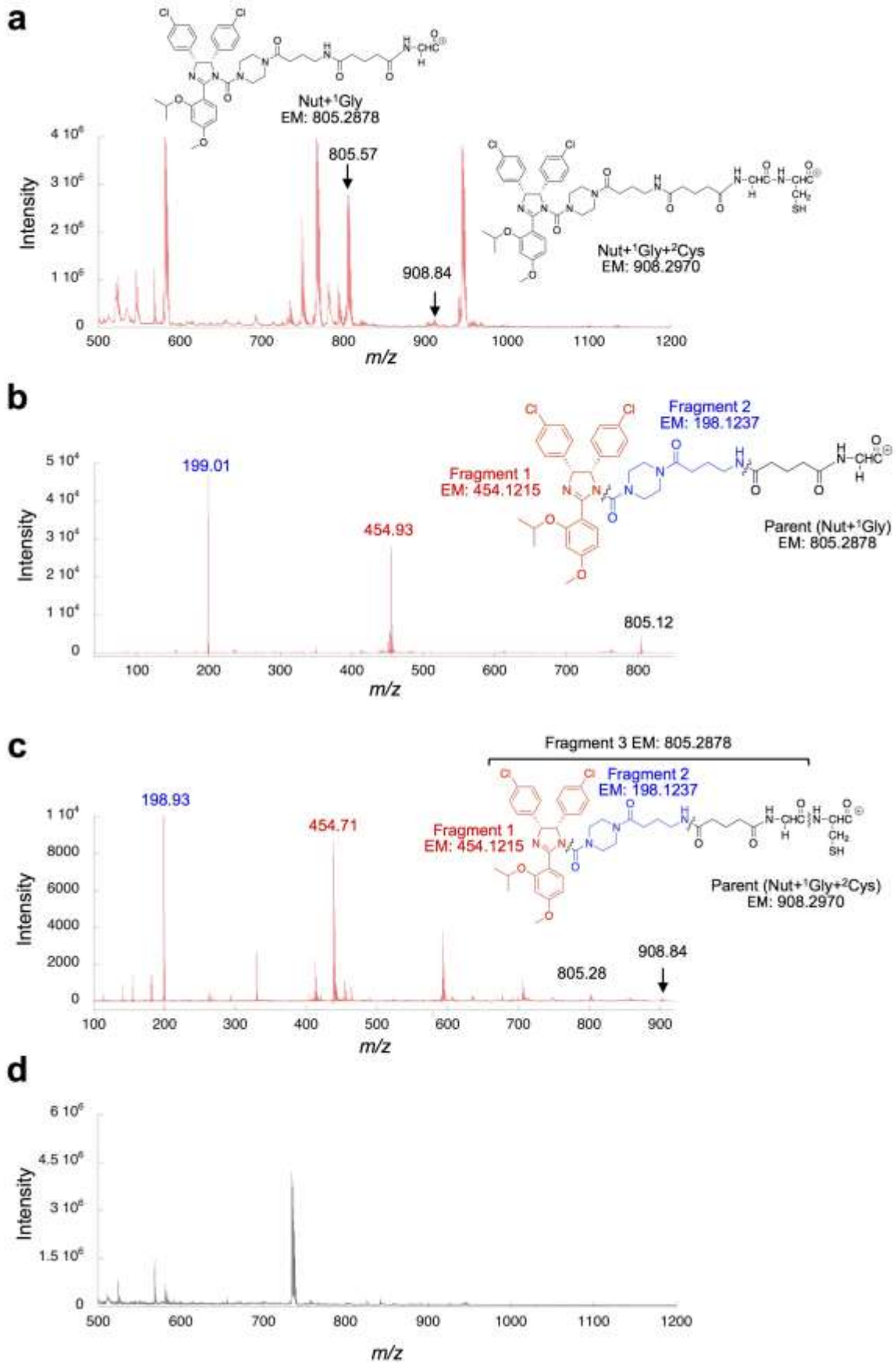


Figure S16 MALDI-TOF/TOF MS analysis of **1**-modified peptide fragments of $HDM2_{1–125}$ (M1G). (a) Fragment mass peaks of T1+**1** (parent MS: 4616.1 Da) and (b) T5+**1** (parent MS: 1415.0 Da) shown in Figure S15b. *, the modification site of **1**, **, carbamidomethylated cysteine. In (a), y34 and b2 ions were detected, indicating that the modification site should be Gly1 (N-terminal amine) or Cys2. There is no possibility that the Cys2 is modified with **1** because it is carbamidomethylated by iodoacetamide before tryptic digestion. Therefore, the N-terminal amino group was identified as one of the modification sites of **1**. In (b), a2 ion, corresponding to **1**-modified Leu-Tyr, was observed. Since leucine does not have any reactive group, the tyrosine should be modified with **1**. This result is also supported by the facts that: (i) two fragments bearing the Nutlin moiety were observed, indicating the parent MS (1415.0 Da) is derived from the peptide modified with **1**; (ii) y3 (DEK) and y1 ion (K) was detected,

suggesting that the DEK sequence is not modified with **1**. Thus, Tyr67 was identified as another modification site of **1**.


a
Nut+¹Gly
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805.57
908.84
Nut+¹Gly+²Cys
EM: 908.2970
Intensity
m/z
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454.93
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Fragment 1
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Fragment 2
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m/z
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805.28
908.84
Fragment 1
EM: 454.1215
Fragment 2
EM: 198.1237
Parent (Nut+¹Gly+²Cys)
EM: 908.2970
Intensity
m/z
d
Intensity
m/z

Figure S17 MALDI-in-source decay (ISD) measurements of **1**-modified $HDM2_{1–125}$ (M1G) for N-terminal structure analysis. (a) ISD spectrum of **1**-modified $HDM2_{1–125}$ (M1G) in the mass range from *m*/*z*-value 500 to 1200. Two fragment mass peaks corresponding to Nutlin-modified N-terminal glycine (Nut+^{1}Gly) and Nutlin-modified N-terminal glycine-cysteine (Nut+^{1}Gly+^{2}Cys) were observed. (b, c) MALDI-ISD with the subsequent TOF/TOF-MS analyses of (b) Nut+^{1}Gly and (c) Nut+^{1}Gly+^{2}Cys. In both cases, two fragments peaks (fragment 1 and 2) of Nutlin-3 were observed, proving that these parent mass ions are derived from compounds containing Nutlin-3 moiety. In addition, a fragment ion that comprises Nutlin-3 and glycine (fragment 3) was observed in (c), which also indicates the N-terminal modification of **1**. (d) ISD spectrum of native (unmodified) $HDM2_{1–125}$ (M1G) in the mass range from *m*/*z*-value 500 to 1200.

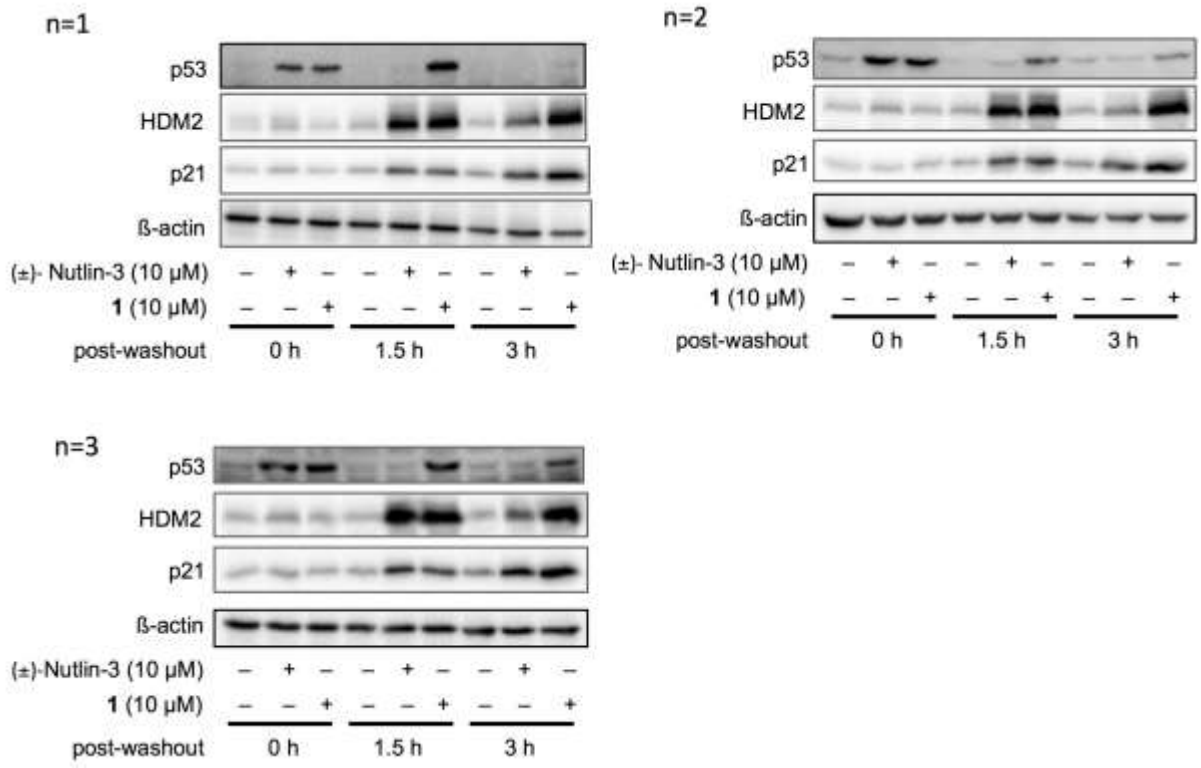

Figure S18 Biological triplicates of western blotting analysis of p53 activation and up-regulation of HDM2 and p21.

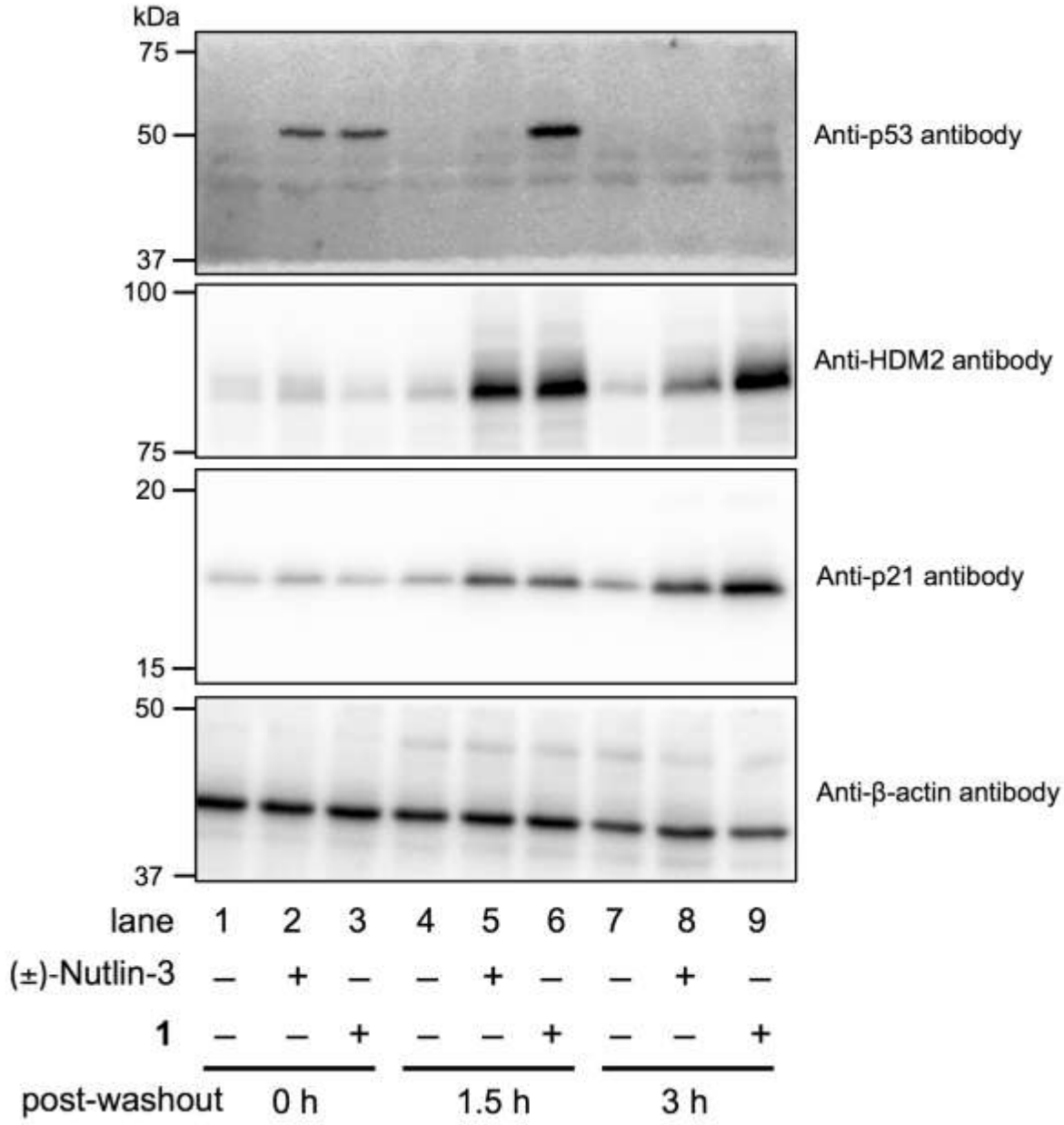

Figure S19 Western blotting analysis of p53 activation and up-regulation of the HDM2 and p21 (Figure 4b in the main text) with molecular weight markers.

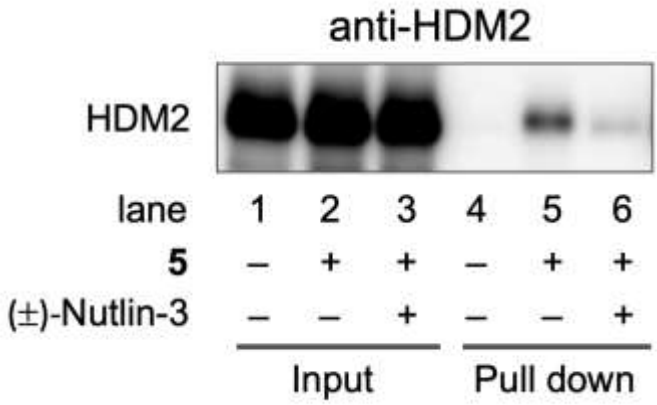

Figure S20 Pull down analysis of HDM2 labeling with **5**. SJSA1 cells were incubated in culture medium containing (±)-Nutlin-3 (10 μM) to induce expression of HDM2. After carefully washing with culture medium, the cells were treated with **5** (1 μM) in the absence or presence of (±)-Nutlin-3 (10 μM) (competitor) for 1 h. After cell lysis, copper-mediated azide-alkyne cycloaddition (CuAAC) was performed to conjugate azide-PEG3-desthiobiotin. The desthiobiotin-labeled proteins were enriched by Neutravidin beads and subsequently analyzed by Western blotting with anti-HDM2 antibody. Endogenous HDM2 was pull-downed in the sample treated with **5**, and the signal was reduced by co-incubation of (±)-Nutlin-3 in the labeling process, indicating affinity-driven covalent labeling of intracellular HDM2 with **5**.

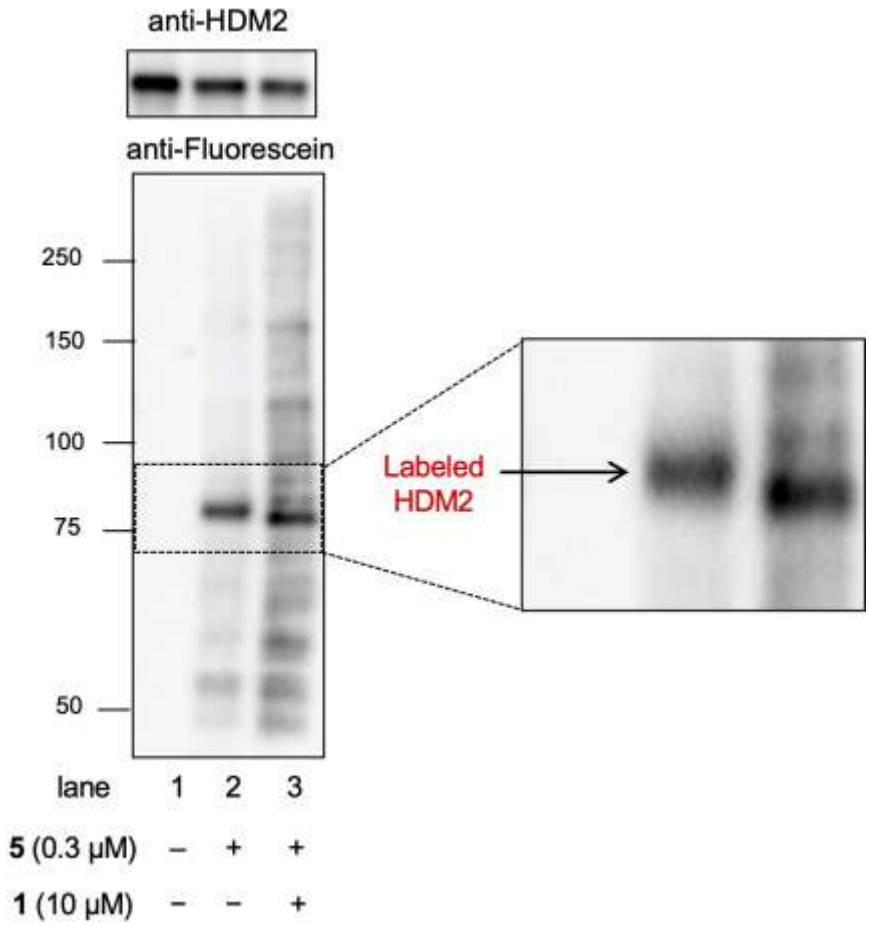

Figure S21 Western blotting analysis of the labeling reaction of endogenous HDM2 with **5** in live SJSA1 cells. The cells were treated with **5** (0.3 μM) in the absence or presence of **1** (10 μM) for 1 h at 37 °C in medium (pH 7.4). After washing, the cells were lysed and analyzed by western blotting using anti-HDM2 and anti-fluorescein antibodies. A clear band corresponding to the labeled HDM2 (indicated by a black arrow) was observed in lane 2. This band was disappeared in the presence of **1** (lane 3), although the background signal of labeling with **5** was elevated throughout the lane because of the cytotoxicity of **1**. This result shows that **1** competitively binds to p53 binding pocket of HDM2 inside cells.

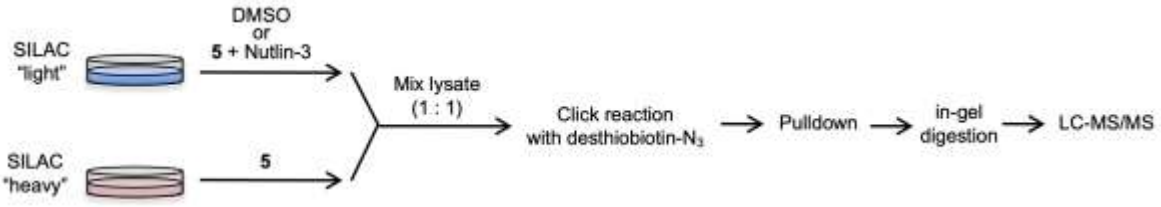

Figure S22 Workflow for SILAC-based quantitative LC-MSMS analysis of proteins labeled with **5**. SJSA1 cells grown in isotopically "light" or "heavy" medium were treated with (±)-Nutlin-3 (10 μM, 4 h). After removal of (±)-Nutlin-3 by washing, "light" amino acid-labeled cells were treated with DMSO or **5** (1 μM) in the presence of (±)-Nutlin-3 (10 μM) for 1 h, whereas "heavy" amino acid-labeled cells were treated with **5** (1 μM). Each cell population was lysed and combined in a 1:1 ratio. The **5**-modified proteins were conjugated to azide-PEG3-desthiobiotin and purified with Neutravidin beads. Enriched proteins were then subjected to in-gel trypsinization and the resultant peptides were analyzed by LC-MS/MS.

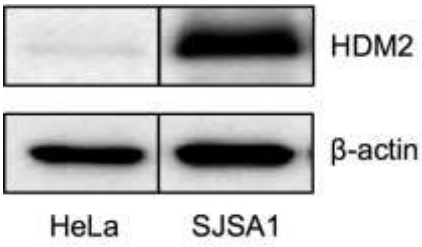

Figure S23 Different expression levels of HDM2 in SJSA1 and HeLa cells. Cell lysates from these cells were subjected to western blotting analysis with anti-HDM2 and anti-β-actin antibodies. This analysis was performed on the same PVDF membrane. It was confirmed that SJSA1 cells ~10-fold overexpress HDM2 compared to HeLa cells. Also, it has been reported that mRNA expression levels of HDM2 do not vary significantly among HeLa, MCF7, and A431 cells.[S2]

Experimental Section

General materials and methods for the biochemical/biological experiments

Unless otherwise noted, all proteins/enzymes and reagents were obtained from commercial suppliers (Sigma-Aldrich, Tokyo Chemical Industry (TCI), Fujifilm Wako Pure Chemical Corporation, Sasaki Chemical, Bio-Rad, Thermo Fisher Scientific, or Watanabe Chemical Industries) and used without further purification. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) spectra were recorded on an Autoflex III, Ultraflex III or UltrafleXtreme (Bruker Daltonics) using α-cyano-4-hydroxycinnamic acid (CHCA) as the matrix. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting were carried out using a Bio-Rad Mini-Protean III electrophoresis apparatus. Fluorescence gel images and chemical luminescent signals using Chemi-Lumi one (Nacalai Tesque) or ECL Prime (GE Healthcare) were acquired with an imagequant LAS4000 (Fujifilm) or a FUSION FX (Vilber). Reversed-phase HPLC (RP-HPLC) was carried out on a Hitachi LaChrom L-7100 system equipped with a LaChrom L-7400 UV detector, and a YMC-Pack ODS-A column (5 μm, 250 × 4.6 mm) at a flow rate of 1.0 mL/min. UV detection was at 220 nm. All runs used linear gradients of acetonitrile containing 0.1% TFA (solvent A) and 0.1% aqueous TFA (solvent B).

Kinetic analysis of HDM2 modification

N-terminal domain of HDM2 (300 nM) was incubated with **1** (500–10000 nM) in PBS buffer (pH 7.4) at 37 °C. Aliquots at different time points were taken, and the reaction was immediately quenched using a Ziptip-C4. The residual ratio of nonlabeled protein ($[P]+[PR]/[P]_0$) were determined by MALDI-TOF MS (matrix: CHCA). The pseudo-first order reaction rates (k_{app}) were obtained by fitting the data to equation (2). Kinetic parameters were obtained by fitting the data to equation (3).[S3]

$$\mathrm{P} + \mathrm{R} \underset{}{\overset{K_d}{\rightleftharpoons}} \mathrm{PR} \xrightarrow{k_{inact}} \mathrm{P}^* \qquad (1)$$

$$\frac{[P]+[PR]}{[P]_0} = \exp(-k_{app}t) \quad (2)$$

$$k_{app} = \frac{k_{inact}}{1 + K_d/[R]} \quad (3)$$

where P is a native (non-modified) protein, R is a reagent, PR is a native protein-reagent complex, P* is a modified-protein, K_d is a dissociation constant, and k_{inact} is a rate constant for covalent modification process.

Chemical labeling of HDM2 with 5 in SJSA1 cells

SJSA1 cells (5.0×10^5 cells) were treated with (±)-Nutlin-3 (10 μM) for 4 h at 37 °C. After washing with PBS, the cells were incubated with **5** (0.3 μM) for 1 h in HEPES-modified RPMI-1640 (FBS-free). After labeling, the cells were washed twice with PBS, and lysed with RIPA buffer containing 1% protease inhibitor cocktail set III. The lysed sample was collected and centrifuged (15 200 *g*, 10 min at 4 °C). Click chemistry was performed on each sample using final concentrations of 10 μM fluorescein-azide[S4], 100 μM bathophenanthrolinedisulfonic acid disodium salt (BPAA), 1 mM tris (2-carboxyethyl)phosphine (TCEP) and 1 mM $CuSO_4$. The samples were vortexed at room temperature for 1 h, mixed with 1/4 volume of 5 × sample buffer containing 250 mM DTT, and vortexed at room temperature for 1 h. The samples were analyzed by western blotting using anti-fluorescein antibody (abcam, ab19491, 1:3000), anti-HDM2/MDM2 (CST, #86934, 1:1000) and anti-rabbit IgG-HRP conjugate (CST, #7074 S, 1:5000).

Identification of proteins modified with 5 in live SJSA1 cells

SJSA1 cells were grown in "light" SILAC media (RPMI-1640, Thermo) containing 100 μg/mL of $^{12}C_6$, $^{14}N_2$-Lys (Thermo) and $^{12}C_6$, $^{14}N_4$,-Arg (Thermo) or "heavy" SILAC media containing $^{13}C_6$, $^{15}N_2$-Lys (Cambridge Isotope Laboratories) and $^{13}C_6$, $^{15}N_4$,-Arg (Cambridge Isotope Laboratories) supplemented with 10% dialyzed FBS (Gibco), penicillin (100 units/ml), streptomycin (100 mg/ml), and amphotericin B (250 ng/ml) under a humidified atmosphere of 5% CO_2 in air at 37 °C. The cells were passaged at least five times in the SILAC media

before being used for analysis. The isotope-labeled cells (5.0×10^5 cells) were seeded on 10 cm dishes (8 dishes for each sample) and incubated for 48 h in the SILAC media. Each SJSA1 cells grown in isotopically "light" or "heavy" medium were treated with (±)-Nutlin-3 (10 μM, 4 h). After washing with serum-free SILAC medium, "light" amino acid-labeled cells were treated with DMSO or **5** (1 μM) in the presence of (±)-Nutlin-3 (10 μM) for 1 h, whereas "heavy" amino acid-labeled cells were treated with **5** (1 μM). After labeling reaction, the cells were washed twice with chilled PBS, lysed with RIPA buffer containing 1% protease inhibitor cocktail set III. The lysed sample was collected and centrifuged (15 200 *g*, 10 min at 4 °C). Equal amounts of protein from heavy (3.7 mg) and light (3.7 mg) samples were mixed in a 1 : 1 ratio (total 4 mL). The combined sample was pre-cleared by High capacity Neutravidin beads (500 μL, Thermo) followed by copper-catalyzed azide-alkyne cycloaddition with azide-PEG3-desthiobiotin (100 μM, TCI), BPAA (100 μM), TCEP (1 mM) and $CuSO_4$. (1 mM). The samples were allowed to react for 1 h at room temperature. The samples (4 mL) were cooled and the proteins were precipitated by addition of ice-cold MeOH (8 mL), $CHCl_3$ (2 mL), and PBS (4 mL) followed by vortexing. The phase was separated by centrifugation (5,000 rpm for 10 min), and the resultant protein discs were washed carefully with 1:1 MeOH/$CHCl_3$ (3 x 4 mL) and dried. Protein pellets were re-suspended with sonication in RIPA buffer containing 1% SDS. The solubilized protein solution was diluted to adjust a SDS concentration of 0.1% and enriched over High capacity Neutravidin beads (100 μL, bed volume) for 2 h at room temperature. The beads were washed with 0.1% SDS RIPA buffer (1 mL, 5 times) and proteins were eluted by incubation in 4 mM biotin solution in RIPA buffer (100 μL) for 1 h at 37 °C. The samples were mixed with 1/4 volume of 5 × sample buffer containing 250 mM DTT and vortexed at room temperature for 1 h. The samples were loaded on a 7.5% SDS–PAGE gel (Mini-PROTEAN TGX Gels, BioRad), and run for 10 min at 20 mA (the migration distance of dye front is about 10 mm). Each lane was cut (height, 10 mm), and fixed with 45% methanol/water containing 5% acetic acid for 20 min. The fixed gel was washed with 50% methanol/water and pure water. The gel pieces were dehydrated with acetonitrile, and then swelled with 200 μL of 10 mM DTT in a 50 mM TEAB (triethylamine bicarbonate) buffer (Sigma Aldrich) and heated at 56 °C for 30

min. The DTT solutions were replaced with a 55 mM iodoacetamide solution in 50 mM TEAB buffer and allowed to react for 45 min in the dark. The gel fractions were washed with a 50 mM TEAB buffer, dehydrated in acetonitrile, rehydrated in 50 mM TEAB buffer, and dehydrated in acetonitrile again. The gels were swelled in a 50 mM TEAB buffer containing 10 ng/μL MS Grade Trypsin/Lys-C mix (Promega) and incubated overnight at 37 °C. After the digestion, the supernatant was transferred to a new tube, and 50 μL of an extraction solution (50% acetonitrile, 0.1% TFA) was added to the gel piece. The supernatants were collected after 10 min incubation, and this process was repeated two additional times. Furthermore, 50 μL of acetonitrile (0.1% TFA) was added to the gel piece. The supernatants were collected after 10 min incubation, and this process was repeated two additional times. Extracted peptides were evaporated and reconstituted in 200 μL of a 5% acetonitrile/0.1% TFA mixture and loaded onto a GL-Tip SDB (GL Sciences). The tip was washed with 5% acetonitrile/0.1% TFA mixture, and peptides were eluted by 80% acetonitrile/0.1% TFA. The solvent was removed in vacuo, and the residue was dissolved in 43 μL of a 5% acetonitrile/0.1% TFA mixture. NanoLC–MS/MS analyses were performed on a Q-Exactive mass spectrometer (Thermo Fisher Scientific) and an Ultimate 3000 nanoLC pump (AMR). Samples were automatically injected using PAL system (CTC analytics, Zwingen, Switzerland) into a peptide L-trap column OSD (5 μm) attached to an injector valve for desalinating and concentrating peptides. After washing the trap with MS-grade water containing 0.1% TFA and 2% acetonitrile, the peptides were loaded into a nano HPLC capillary column (C18 packed with the gel particle size of 3 μm, 0.1 x 125 mm, Nikkyo Technos, Tokyo Japan) by switching the valve. The injection volume was 10 μL and the flow rate was 500 nL/min. The mobile phases consisted of (A) 0.5% acetic acid and (B) 0.5% acetic acid and 80% acetonitrile. A two-step linear gradient of 5–45% B in 60 min, 45–95% B in 1 min, 95% B for 20 min was employed. Spray voltages of 2,000 V were applied. The mass scan ranges were m/z 350–1,800, and top ten precursor ions were selected in each MS scan for subsequent MS/MS scans. The normalized collision energy was set to be 30. The raw MS data files were analyzed by Proteome Discoverer 2.2 (Thermo Fisher Scientific) to create peak lists based on the recorded fragmentation spectra. Peptides and proteins were identified

by means of automated database searching using Sequest HT (Thermo Fisher Scientific) against UniprotKB/Swiss-Prot release 2019-05-10 with a precursor mass tolerance of 10 p.p.m., a fragment ion mass tolerance of 0.02 Da, and trypsin specificity that allows for up to three or two missed cleavages. Cysteine carbamidomethylation was set as fixed modifications. Methionine oxidation was allowed as a variable modification. A reversed decoy database search was conducted to set false discovery rates (FDRs) of less than 1% both at peptide and protein levels. Three independent SILAC experiments were performed, in which at least two identified and quantified peptides were considered hit proteins, and proteins detected at least twice in three replicates as identified proteins.

Construction of expression plasmids in mammalian cells

Using the pET28a-HDM2$_{1\text{-}125}$ as a template, cDNAs encoding HA-HDM2$_{1\text{-}125}$, HDM2$_{1\text{-}125}$-HA, and HDM2$_{1\text{-}125}$ (Y67F)-HA were prepared by PCR. These cDNAs were subcloned into the expression vector, pCAGGS (kindly provided by Dr J. Miyazaki, Osaka University, Osaka, Japan) with the restriction enzymes NheI, KpnI, and NotI.

Chemical labeling of transiently expressed HDM2 with 5–7 in HEK293T cells

HEK293T cells (2.0×10^5 cells) were seeded on 35 mm dish and incubated for 20 h at 37 °C under 5% CO_2 atmosphere. The cells were transfected with a plasmid encoding HDM2 using lipofectamine 2000 (Invitrogen) and subjected to labeling experiments after 20 h of the transfection. The cells were incubated with **5–7** (0.3 μM) for 1 h in HEPES-modified DMEM (FBS-free). After labeling, the cells were washed twice with PBS, and lysed with RIPA buffer containing 1% protease inhibitor cocktail set III. The lysed sample was collected and centrifuged (15 200 *g*, 10 min at 4 °C). Click chemistry was performed on each sample using final concentrations of 10 μM fluorescein-azide, 100 μM BPAA, 1 mM TCEP and 1 mM $CuSO_4$. The samples were vortexed at room temperature for 1 h, mixed with 1/4 volume of 5 × sample buffer containing 250 mM DTT, and vortexed at room temperature for 1 h. The samples were analyzed by western blotting using anti-fluorescein antibody, anti-HA-HRP conjugate (CST, #2999S, 1:1000) and anti-rabbit IgG-HRP conjugate.

General materials and methods for organic synthesis

All chemical reagents and solvents were obtained from commercial suppliers (Tokyo Chemical Industry (TCI), Sigma-Aldrich, Fujifilm-Wako Pure Chemical Corporation, Watanabe Chemical Industries, or Kanto Chemical Co., Inc.) and used without further purification. Thin layer chromatography (TLC) was performed on silica gel 60 F254 precoated aluminium sheets (Merck) and visualized by fluorescence quenching, fluorescence by 365 nm excitation, I_2 staining and ninhydrin staining. Chromatographic purification was accomplished using flash column chromatography on silica gel 60 N (neutral, 40–50 μm, Kanto Chemical). ^{1}H NMR spectra were recorded in deuterated solvents on a Varian Mercury 400 (400 MHz) spectrometer and calibrated to tetramethylsilane (= 0 ppm) or residual solvent peak. Multiplicities are abbreviated as follows: s = singlet, brs= broad singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = double doublet. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) spectra were recorded on an Autoflex III instrument (Bruker Daltonics) using α-cyano-4-hydroxycinnamic acid (CHCA) as the matrix. High-resolution mass spectra were measured on an Exactive (Thermo Scientific) equipped with electron spray ionization (ESI). Reversed-phase HPLC (RP-HPLC) was carried out on a Hitachi LaChrom L-7100 system equipped with a LaChrom L-7400 UV detector, and a YMC-Pack ODS-A column (5 μm, 250 × 20 mm) at a flow rate of 9.9 mL/min. UV detection was at 220 nm and 512 nm. All runs used linear gradients of acetonitrile containing 0.1% TFA (solvent A) and 0.1% aqueous TFA (solvent B).

Synthesis

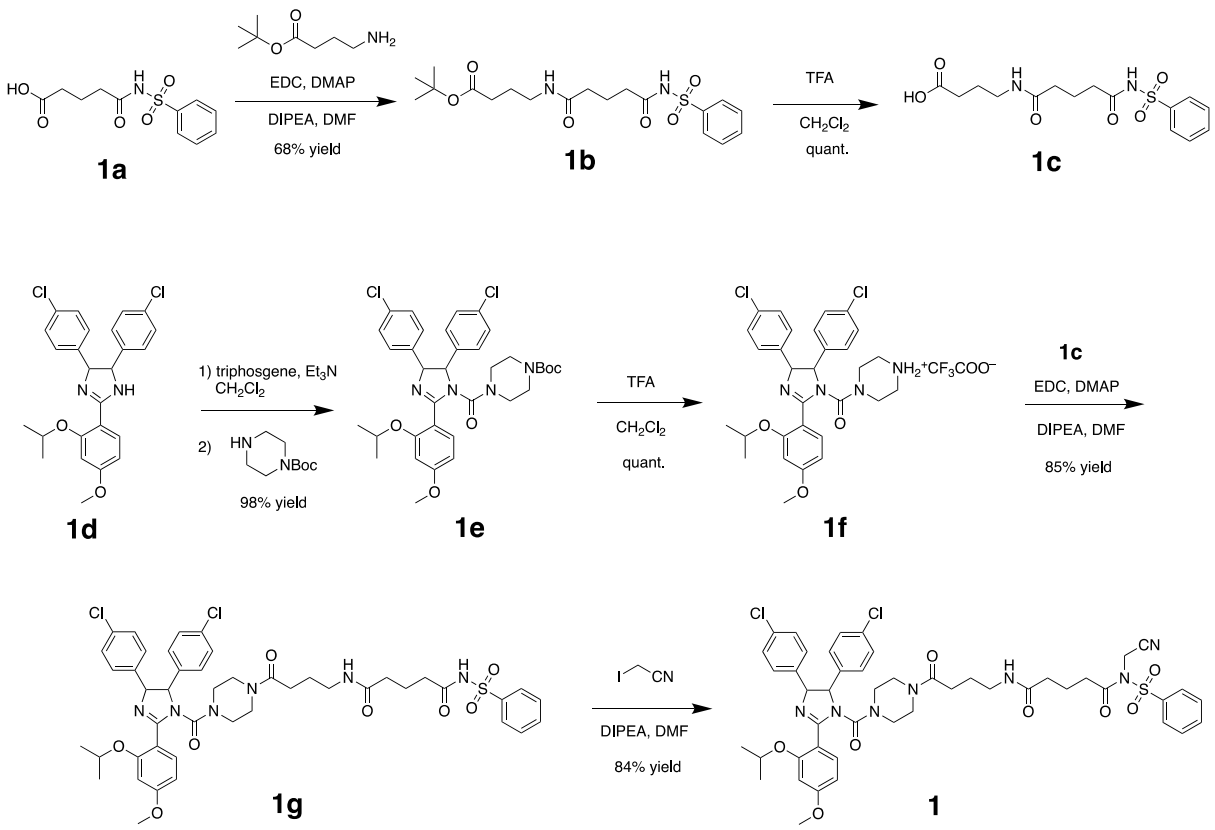

Figure S24 Synthetic scheme of covalent inhibitor **1**.

*Compound **1b***

A solution of H-γ-Abu-OtBu (25 mg, 0.132 mmol), **1a**[S3] (30 mg, 0.110 mmol), *N*,*N*-dimethyl-4-aminopyridine (DMAP) (16 mg, 0.132 mmol), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) (25 mg, 0.132 mmol) and *N*,*N*-diisopropylethylamine (DIPEA) (115 μL, 0.663 mmol) in dry *N*,*N*-dimethylformamide (DMF) (1 mL) was stirred at room temperature for 37 h. The solvent was removed under reduced pressure. The residue was dissolved in ethyl acetate (EtOAc) (30 mL), washed with 10% citric acid aq. (5 mL x2), water (4 mL) and brine (4 mL), dried over $MgSO_4$ and concentrated under reduced pressure. The crude was purified by flash chromatography on silica gel (MeOH:$CHCl_3$ = 1:50 to 1:20) to yield compound **1b** (31 mg, 0.075 mmol, 68%) as colorless oil.

^{1}H NMR (400 MHz, CD_3OD) δ 8.01 (d, J = 7.2 Hz, 2H), 7.69–7.65 (m, 1H), 7.59–7.56 (m, 2H), 3.14 (t, J = 7.2 Hz, 2H), 2.27–2.21 (m, 4H), 2.13 (t, J = 7.2 Hz, 2H), 1.78 (quint, J = 7.2 Hz, 2H), 1.71 (quint, J = 7.2 Hz, 2H), 1.44 (s, 9H).

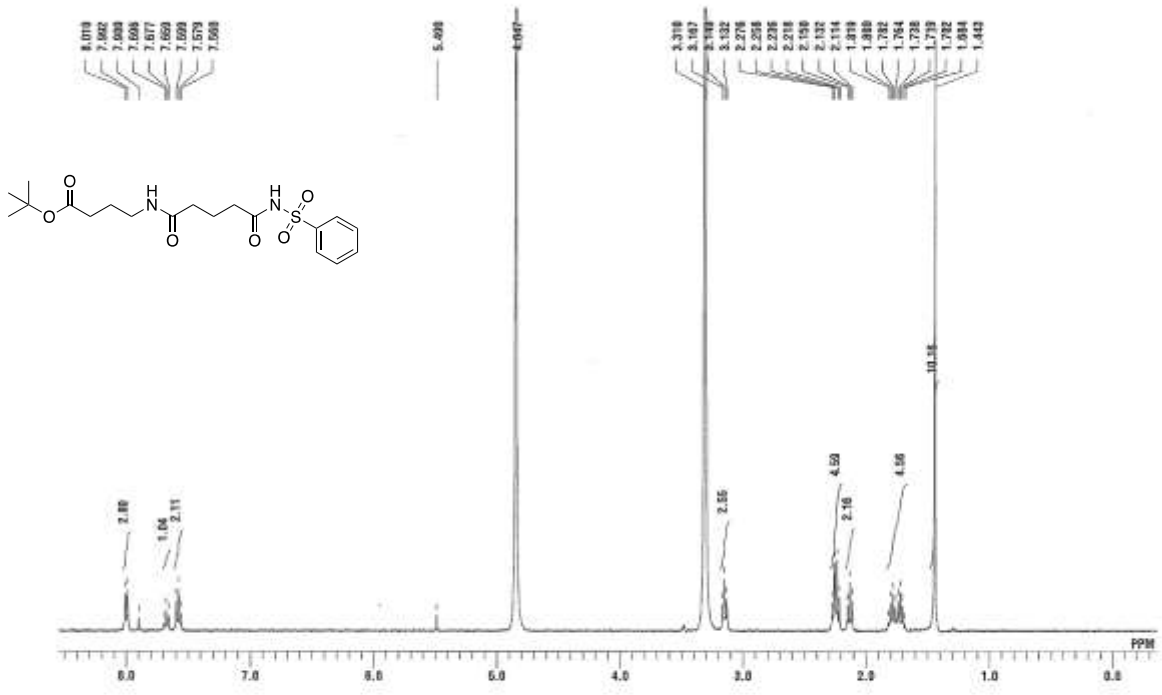

Figure S25 ^{1}H-NMR spectrum of **1b**.

*Compound **1c***

To a solution of **1b** (31 mg, 0.075 mmol) in CH_2Cl_2 (1 mL) was added trifluoroacetic acid (TFA) (0.5 mL) at room temperature. The reaction solution was allowed to stir for 30 min. TFA was removed by twice co-evaporation with toluene (1 mL). The product was used in the next step without further purification.

*Compound **1e***

To a solution of **1d**[S5] (180 mg, 0.396 mmol) and triethylamine (Et_3N) (0.39 mL, 2.79 mmol) dissolved in CH_2Cl_2 (30 mL) at 0 ºC was added a solution of bis(trichloromethyl)carbonate (195 mg, 0.657 mmol) in CH_2Cl_2 (30 mL). The reaction mixture was stirred at 0 ºC for 30 min and solvent was removed. The residue was dissolved in CH_2Cl_2 (30 mL), and a solution

of tert-butoxycarbonyl piperazine (1.1 g, 5.94 mmol) in CH_2Cl_2 (30 mL) was added. The resulting mixture was warmed up to room temperature and stirred for 30 min. The reaction was quenched with 10% citric acid aq. (20 mL), and extracted with CH_2Cl_2. The organic phase was washed with sat. $NaHCO_3$ aq. (20 mL) and brine (10 mL), dried over $MgSO_4$, and concentrated under reduced pressure. The crude was purified by flash chromatography on silica gel (EtOAc:hexane = 1:2 to 2:1) to yield **1e** (259 mg, 0.388 mmol, 98%) as a white solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.51 (d, J = 8.4 Hz, 1H), 7.08 (d, J = 8.4 Hz, 2H), 7.02 (d, J = 8.4 Hz, 2H), 6.94 (d, J = 8.4 Hz, 2H), 6.85 (d, J = 8.4 Hz, 2H), 6.54 (dd, J = 8.4, 2.0 Hz, 1H), 6.50 (d, J = 2.0 Hz, 1H), 5.59 (d, J = 9.6 Hz, 1H), 5.45 (d, J = 9.6 Hz, 1H), 4.61 (quint, J = 6.0 Hz, 1H), 3.85 (s, 3H), 3.07–3.06 (m, 4H), 2.97–2.95 (m, 4H), 1.41 (s, 9H), 1.39 (d, J = 6.0 Hz, 3H), 1.35 (d, J = 6.0 Hz, 3H).

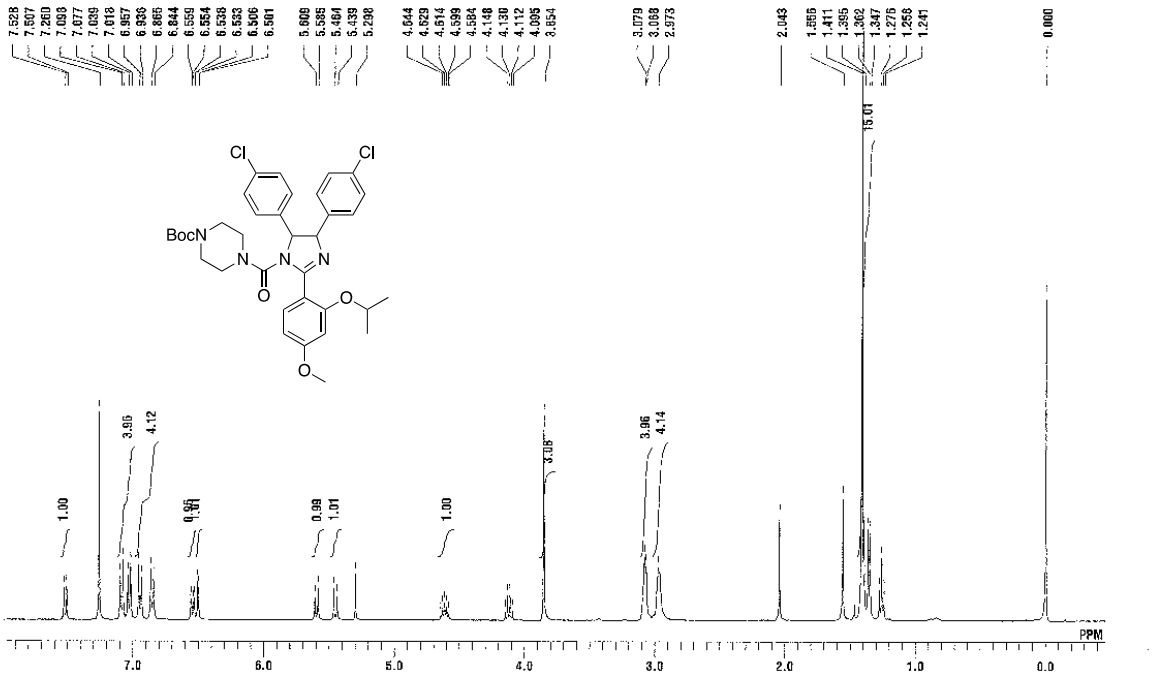

Figure S26 ^{1}H-NMR spectrum of **1e**.

*Compound **1f***

To a solution of **1e** (41 mg, 0.062 mmol) in CH_2Cl_2 (1 mL) was added TFA (0.5 mL) at room temperature. The reaction solution was allowed to stir for 30 min. TFA was removed by twice co-evaporation with toluene (1 mL) to afford **1f** (42 mg, 0.062 mmol, quant.). The product was used in the next step without further purification.

*Compound **1g***

To a stirred solution of **1f** (42 mg, 0.062 mmol) and **1c** (26 mg, 0.075 mmol) in dry DMF (2 mL) was added EDC (14 mg, 0.075 mmol), DMAP (9.1 mg, 0.075 mmol) and DIPEA (64 µL, 0.37 mmol). The mixture was stirred at room temperature for 24 h. The solvent was removed under reduced pressure. The residue was dissolved with $CHCl_3$ (50 mL), washed with 10% citric acid aq. (10 mL) and brine (4 mL), dried over $MgSO_4$ and concentrated under reduced pressure. The resultant was purified by flash chromatography on silica gel (MeOH:$CHCl_3$ = 1:50 to 1:10) to yield **1g** (48 mg, 0.053 mmol, 85%) as a white solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.03 (d, J = 4.0 Hz, 2H), 7.61–7.51 (m, 4H), 7.08 (d, J = 8.4 Hz, 2H), 7.02 (d, J = 8.4 Hz, 2H), 6.93 (d, J = 8.4 Hz, 2H), 6.86 (d, J = 8.4 Hz, 2H), 6.55 (d, J = 8.4 Hz, 1H), 6.50 (s, 1H), 6.35 (brs, 1H), 5.59 (d, J = 10 Hz, 1H), 5.47 (d, J = 10 Hz, 1H), 4.61 (quint, J = 6.0 Hz, 1H), 3.84 (s, 3H), 3.26–3.25 (m, 10H), 2.36–2.16 (m, 6H), 1.87–1.79 (m, 4H), 1.39 (d, J = 5.6 Hz, 3H), 1.34 (d, J = 5.6 Hz, 3H).

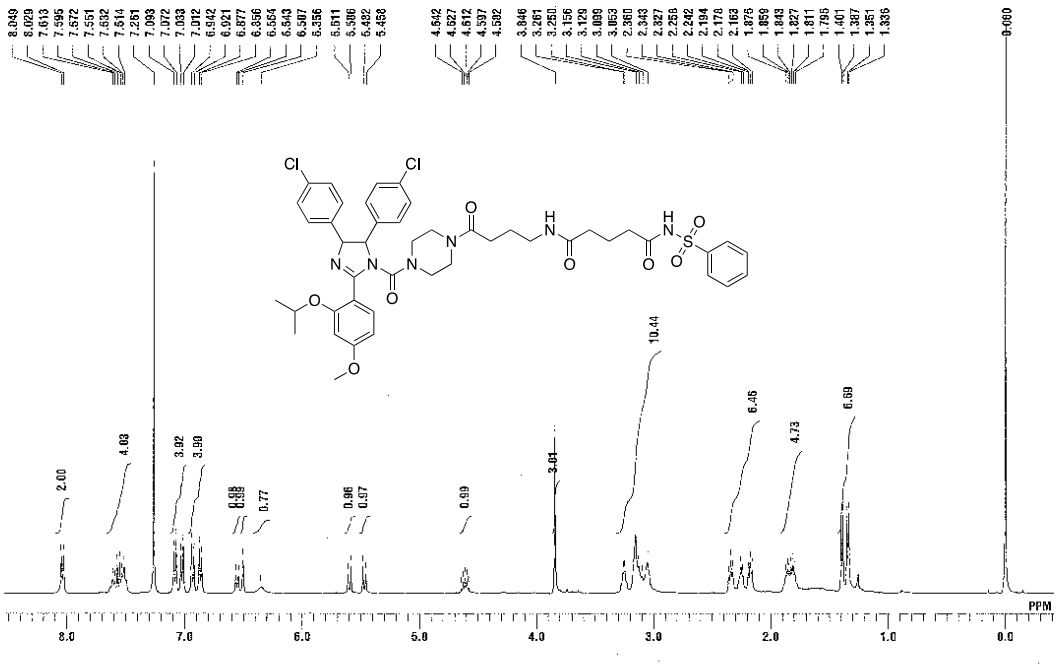

Figure S27 ^{1}H-NMR spectrum of **1g**.

*Covalent inhibitor **1***

To a solution of **1g** (16 mg, 0.0176 mmol) in dry DMF (0.8 mL) was added DIPEA (61 µL, 352 µmol) and iodoacetonitrile (12.8 µL, 176 µmol). The mixture was stirred at room temperature for 24 h in the dark. The solvent was removed under reduced pressure. The residue was dissolved with $CHCl_3$ (50 mL), washed with 10% citric acid aq. (10 mL) and brine (10 mL), dried over $MgSO_4$ and concentrated under reduced pressure. The resultant was purified by flash chromatography on silica gel (MeOH:$CHCl_3$ = 1:40 to 1:20) to yield **1** (14.1 mg, 0.014 mmol, 84%) as a white solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.97 (d, J = 7.2 Hz, 2H), 7.73 (dd, J = 7.2, 7.2 Hz, 1H), 7.63 (dd, J = 7.2, 7.2 Hz, 2H), 7.53 (dd, J = 7.2, 7.2 Hz, 1H), 7.08 (d, J = 8.4 Hz, 2H), 7.03 (d, J = 8.4 Hz, 2H), 6.93 (d, J = 8.4 Hz, 2H), 6.86 (d, J = 8.4 Hz, 2H), 6.55 (dd, J = 8.4, 2.0 Hz, 1H), 6.50 (s, 1H), 6.00 (brs, 1H), 5.58 (d, J = 9.6 Hz, 1H), 5.46 (d, J = 9.6 Hz, 1H), 4.74 (s, 2H), 4.61 (quint, J =6.0 Hz, 1H), 3.84 (s, 3H), 3.24–3.01 (m, 10H), 2.75 (t, J = 7.2 Hz, 2H), 2.27 (t, J = 6.4 Hz, 2H), 2.12 (t, J = 7.2 Hz, 2H), 1.89 (quint, J = 6.8 Hz, 2H), 1.78 (quint, J = 6.4 Hz, 2H), 1.39 (d, J = 6.0 Hz, 3H), 1.34 (d, J =6.0 Hz, 3H).

^{13}C NMR (150 MHz, $CDCl_3$) δ 171.9, 171.6, 171.0, 163.0, 160.4, 157.3, 155.4, 138.0, 136.1, 135.2, 134.9, 133.1, 132.9, 132.1, 129.9, 129.3, 128.5, 128.1, 128.0, 127.7, 114.7, 113.6, 104.5, 100.2, 77.2, 77.0, 76.8, 71.4, 71.0, 69.2, 55.6, 46.1, 45.6, 44.7, 40.9, 39.4, 35.1, 34.9, 33.1, 30.8, 24.2, 22.2, 22.1, 20.3

HRMS (ESI) calcd for $[C_{47}H_{52}Cl_2N_7O_8S_1]^+$ $(M+H)^+$: *m/z* 944.2970, found 944.2950.

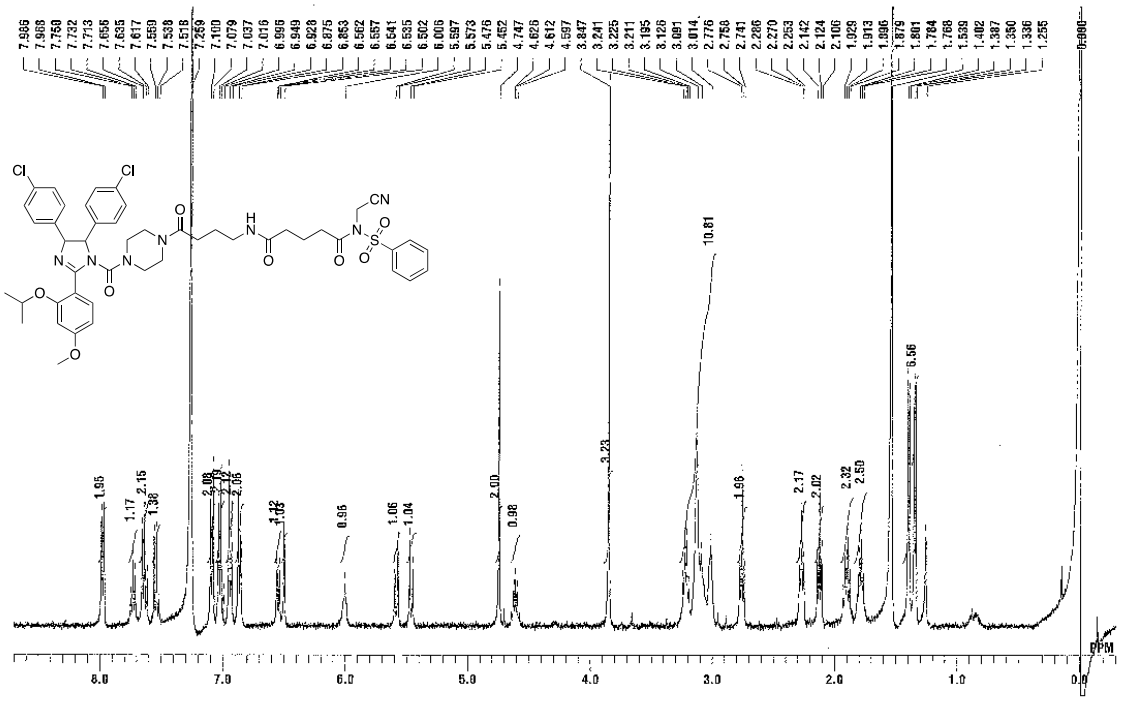

Figure S28 ^{1}H-NMR spectrum of **1**.

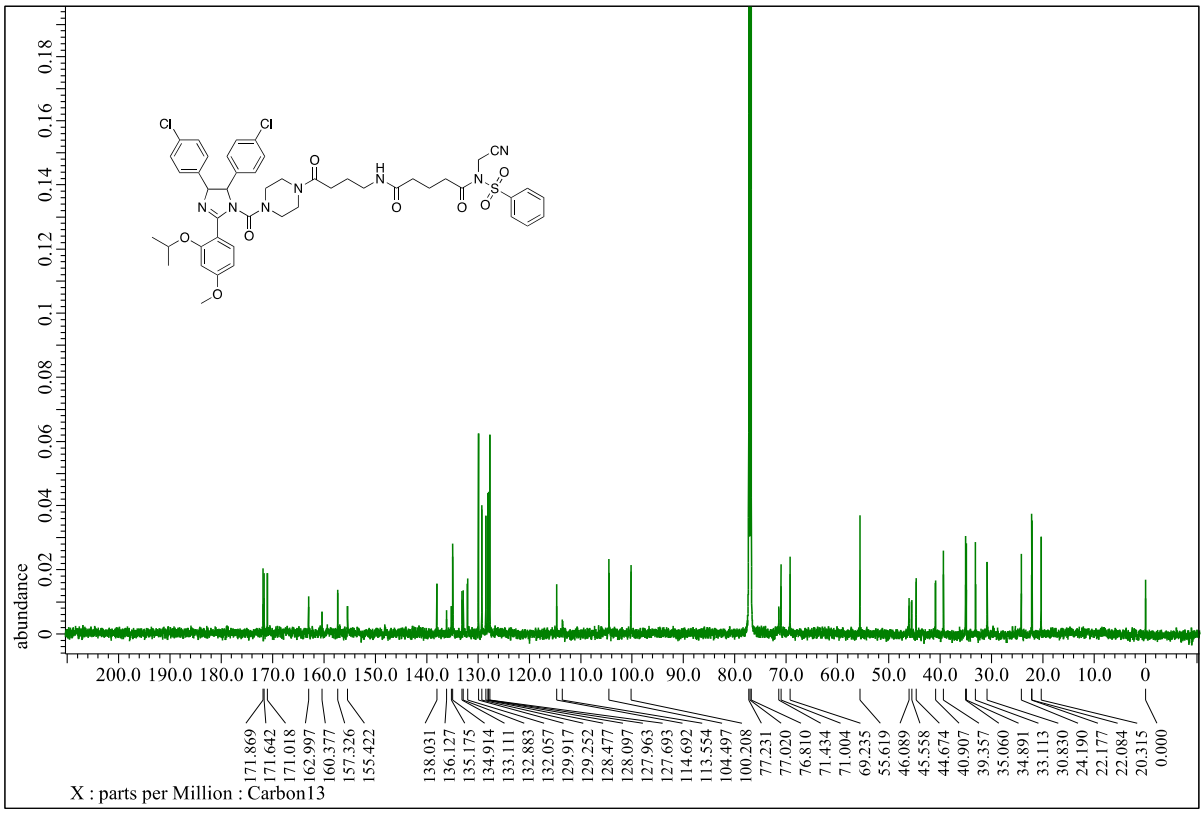

Figure S29 ^{13}C-NMR spectrum of **1**.

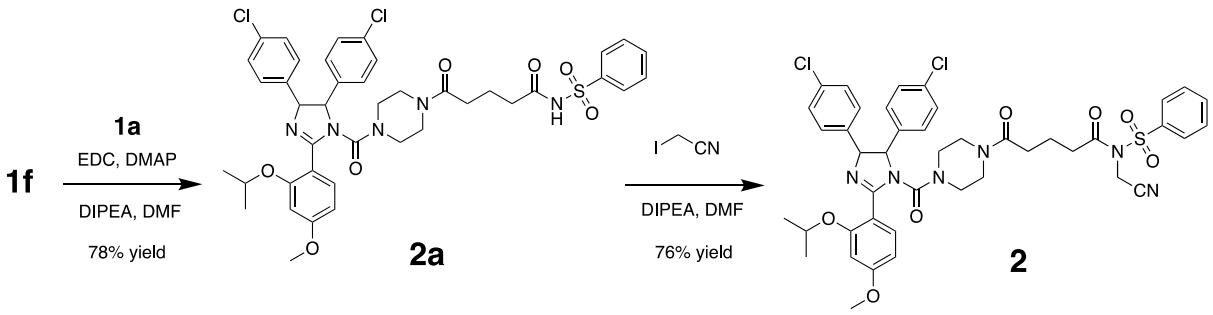

Figure S30 Synthetic scheme of covalent inhibitor **2**.

*Compound **2a***

To a solution of **1f** (10 mg, 0.015 mmol) in dry DMF (1 mL) was added **1a** (4.8 mg, 0.018 mmol), EDC (3.4 mg, 0.018 mmol), DMAP (2.1 mg, 0.018 mmol) and DIEA (15.6 µL, 0.090 mmol). The mixture was stirred at room temperature for 24 h. The solvent was removed under

reduced pressure. The residue was dissolved with $CHCl_3$ (50 mL), washed with 10% citric acid aq. (10 mL) and brine (10 mL), dried over $MgSO_4$ and concentrated under reduced pressure. The resultant was purified by flash chromatography on silica gel (MeOH:$CHCl_3$ = 1:50 to 1:10) to yield **2a** (11 mg, 0.013 mmol, 78%) as a white solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.02 (d, J = 7.2 Hz, 2H), 7.63–7.49 (m, 4H), 7.09 (d, J = 8.4 Hz, 2H), 7.03 (d, J = 8.4 Hz, 2H), 6.94 (d, J = 8.4 Hz, 2H), 6.86 (d, J = 8.4 Hz, 2H), 6.56 (dd, J = 8.4, 2.0 Hz, 1H), 6.50 (d, J = 2.0 Hz, 1H), 5.59 (d, J = 9.6 Hz, 1H), 5.47 (d, J = 9.6 Hz, 1H), 4.61 (quint, J = 6.0 Hz, 1H), 3.84 (s, 3H), 3.13–2.99 (m, 8H), 2.29–2.23 (m, 4H), 1.80 (quint, J = 6.4 Hz, 2H), 1.40 (d, J = 6.0 Hz, 3H), 1.35 (d, J = 6.0 Hz, 3H).

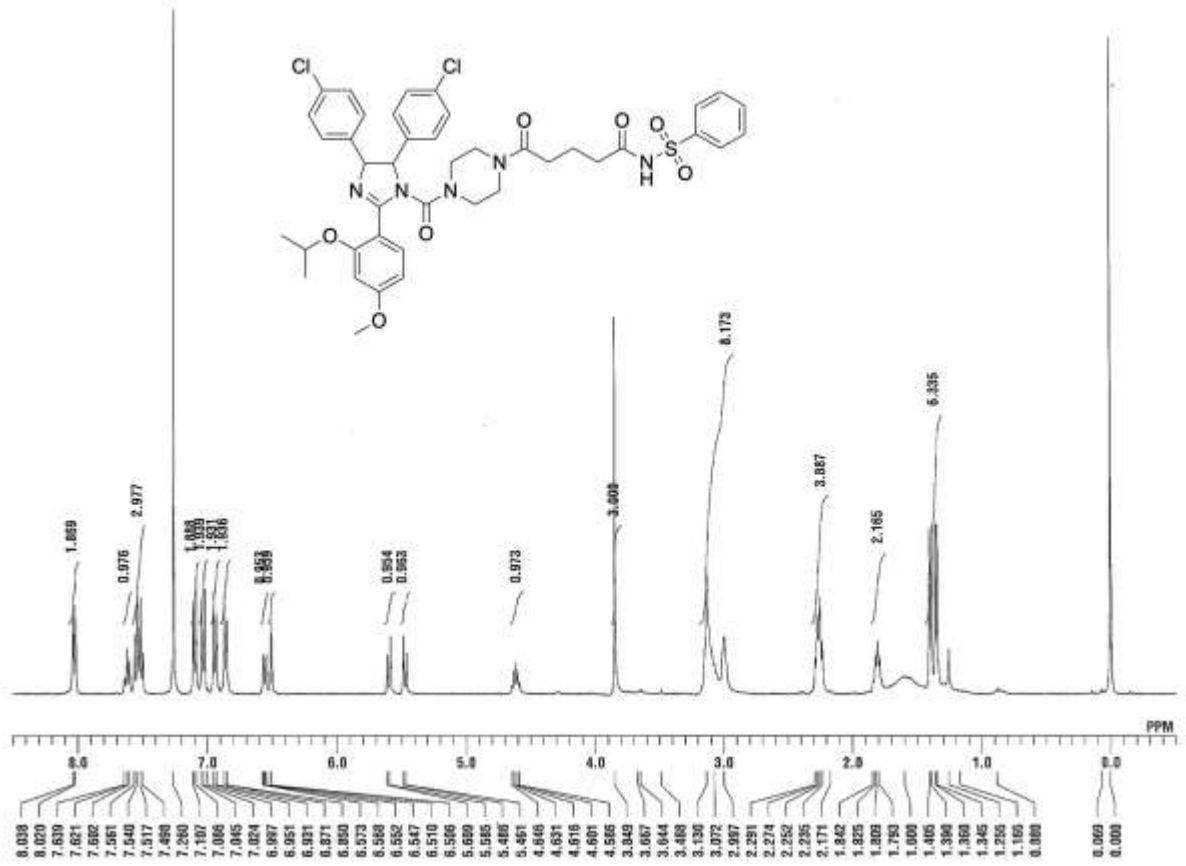

Figure S31 ^{1}H-NMR spectrum of **2a**.

*Compound **2***

To a solution of **2a** (11 mg, 0.0134 mmol) in dry DMF (0.5 mL) was added DIEA (46 µL, 268 µmol) and iodoacetonitrile (9.7 µL, 134 µmol). The mixture was stirred at room temperature for 24 h in the dark. The solvent was removed under reduced pressure. The residue was dissolved with $CHCl_3$ (30 mL), washed with 10% citric acid aq. (10 mL) and brine (4 mL), dried over $MgSO_4$ and concentrated under reduced pressure. The resultant was purified by flash chromatography on silica gel (MeOH:$CHCl_3$ = 1:50 to 1:30) to yield **2** (8.8 mg, 0.010 mmol, 76%) as a beige solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.96 (d, J = 7.2 Hz, 2H), 7.72 (dd, J = 7.6, 7.6 Hz, 1H), 7.62 (dd, J = 7.6, 7.6 Hz, 2H), 7.53 (d, J = 8.4 Hz, 1H), 7.09 (d, J = 8.8 Hz, 2H), 7.03 (d, J = 8.8 Hz, 2H), 6.94 (d, J = 8.8 Hz, 2H), 6.86 (d, J = 8.8 Hz, 2H), 6.55 (dd, J = 8.8, 2.4 Hz, 1H), 6.50 (d, J = 2.4 Hz, 1H), 5.59 (d, J = 9.6 Hz, 1H), 5.46 (d, J = 9.6 Hz, 1H), 4.74 (s, 2H), 4.61 (quint, J = 6.0 Hz, 1H), 3.84 (s, 3H), 3.11–2.99 (m, 8H), 2.76 (t, J = 6.4 Hz, 2H), 2.20 (t, J = 7.2 Hz, 2H), 1.86 (quint, J = 7.2 Hz, 2H), 1.40 (d, J = 6.0 Hz, 3H), 1.35 (d, J = 6.0 Hz, 3H).

^{13}C NMR (150 MHz, $CDCl_3$) δ 171.8, 170.2, 162.9, 160.3, 157.3, 155.4, 138.0, 136.2, 135.2, 134.9, 133.1, 132.9, 132.0, 129.9, 129.3, 128.5, 128.1, 128.0, 127.7, 114.6, 113.6, 104.4, 100.2, 71.5, 70.9, 69.2, 55.6, 46.0, 45.6, 44.6, 40.8, 35.0, 33.1, 31.5, 22.2, 22.1, 19.8.

HRMS (ESI) calcd for $[C_{43}H_{45}Cl_2N_6O_7S_1]^+$ $(M+H)^+$: *m/z* 859.2442, found 859.2436.

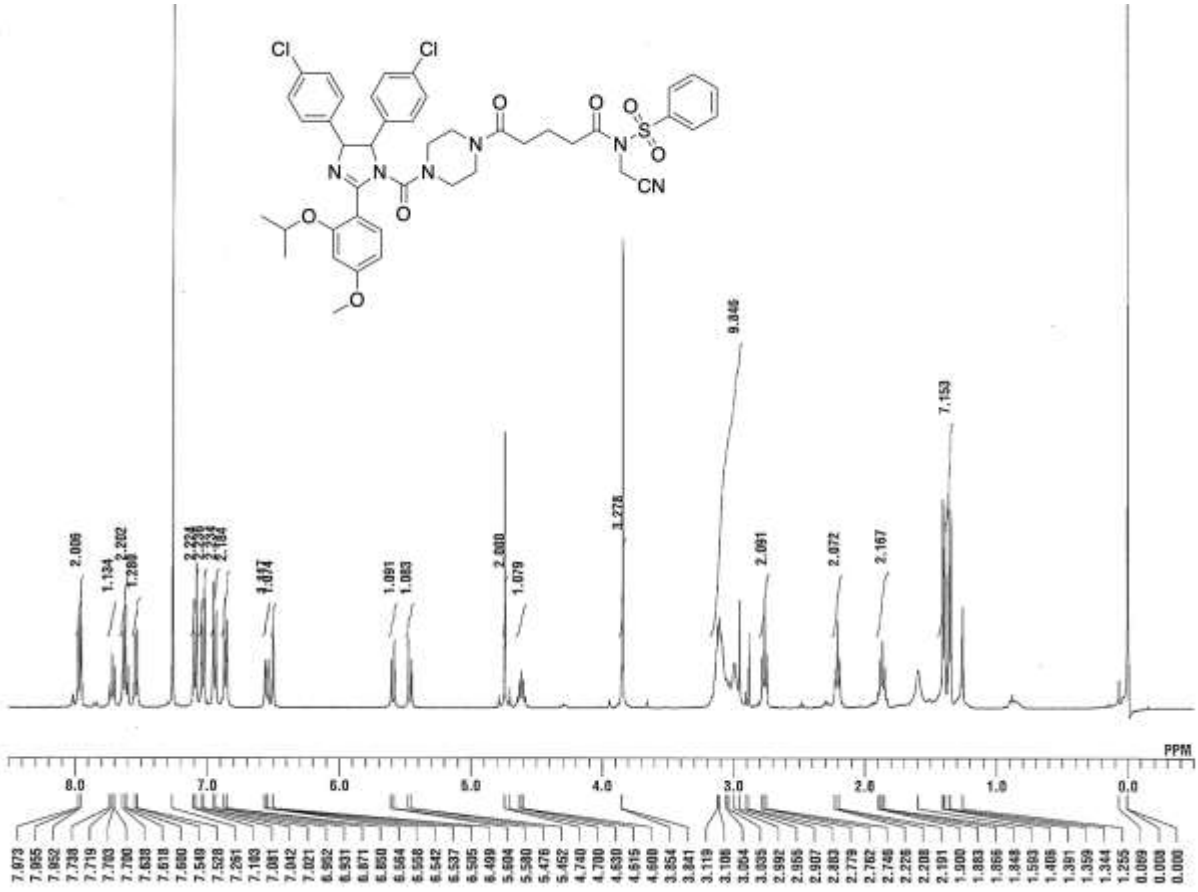

Figure S32 ^{1}H-NMR spectrum of **2**.

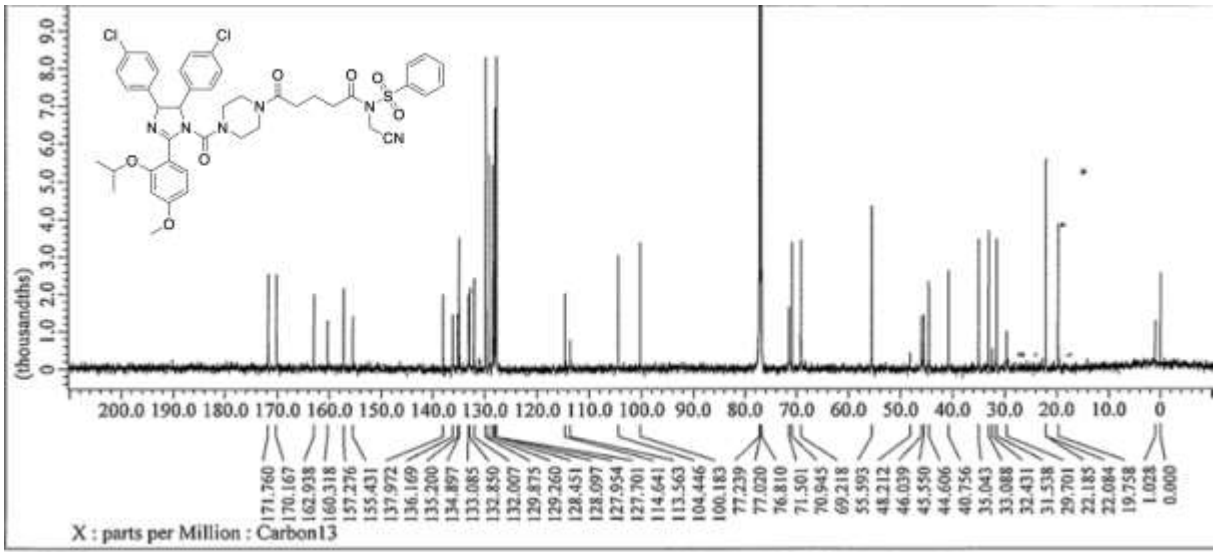

Figure S33 ^{13}C-NMR spectrum of **2**.

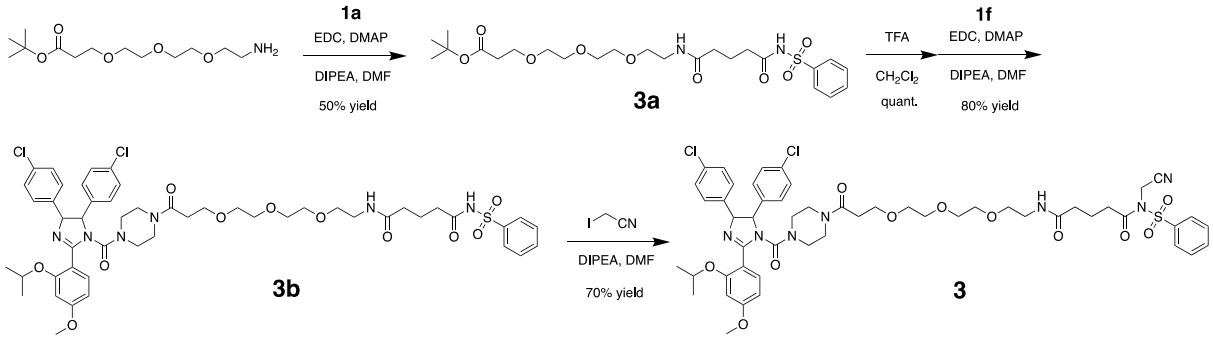

Figure S34 Synthetic scheme of covalent inhibitor **3**.

*Compound **3a***

A solution of 1,1-Dimethylethyl 3-[2-[2-(2-aminoethoxy)ethoxy]ethoxy]propanoate (36 mg, 0.132 mmol), **1a** (30 mg, 0.110 mmol), DMAP (16 mg, 0.132 mmol), EDC (25 mg, 0.132 mmol) and DIEA (115 μL, 0.663 mmol) in dry DMF (1 mL) was stirred at room temperature for 37 h. The solvent was removed under reduced pressure. The residue was dissolved in EtOAc (30 mL), washed with 10% citric acid aq. (5 mL x2), water (4 mL) and brine (4 mL), dried over $MgSO_4$ and concentrated under reduced pressure. The resultant was purified by flash chromatography on silica gel (MeOH:$CHCl_3$ = 1:50 to 1:20) to yield compound **3a** (29 mg, 0.054 mmol, 50%) as colorless oil.

^{1}H NMR (400 MHz, CD_3OD) δ 8.00 (d, J = 7.6 Hz, 2H), 7.68 (dd, J = 7.6 Hz, 7.2 Hz, 1H) 7.60–7.55 (m, 2H), 3.69 (t, J = 6.0 Hz, 2H), 3.61–3.54 (m, 10H), 3.51 (t, J = 5.6 Hz, 2H), 2.47 (t, J = 6.4 Hz, 2H), 2.26 (t, J = 7.2 Hz, 2H), 2.16 (t, J = 7.2 Hz, 2H), 1.79 (quint, J = 7.2 Hz, 2H), 1.45 (s, 9H).

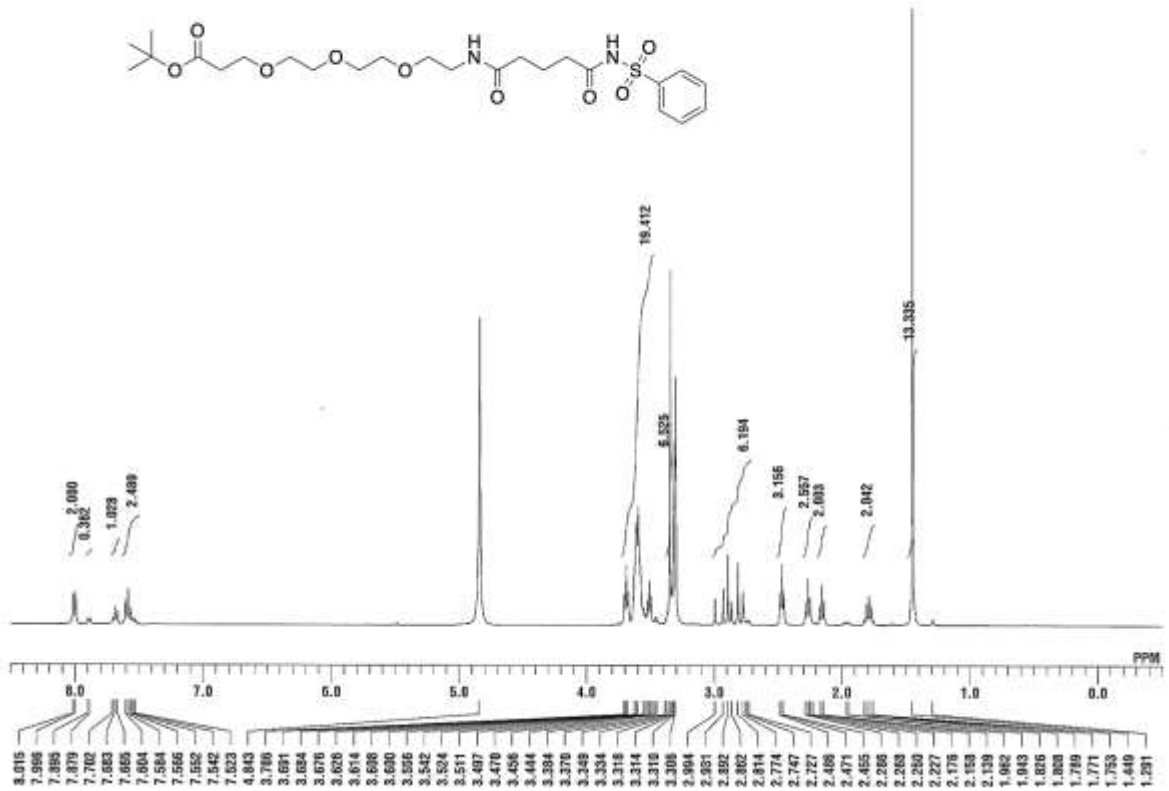

Figure S35 ^{1}H-NMR spectrum of **3a**.

*Compound **3b***

To a solution of **3a** (29 mg, 0.054 mmol) in CH_2Cl_2 (1 mL) was added TFA (0.5 mL) at room temperature. The reaction solution was allowed to stir for 30 min. TFA was removed by twice co-evaporation with toluene (1 mL). To a stirred solution of this residues in dry DMF (2 mL) was added **1f** (30 mg, 0.045 mmol), EDC (10 mg, 0.054 mmol), DMAP (6.5 mg, 0.054 mmol) and DIEA (47 µL, 0.27 mmol). The mixture was stirred at room temperature for 24 h. The solvent was removed under reduced pressure. The residue was dissolved with $CHCl_3$ (50 mL), washed with 10% citric acid aq. (10 mL) and brine (5 mL), dried over $MgSO_4$ and concentrated under reduced pressure. The resultant was purified by flash chromatography on silica gel (MeOH:$CHCl_3$ = 1:50 to 1:20) to yield **3b** (37 mg, 0.036 mmol, 80%) as a white solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.04 (d, J = 7.6 Hz, 2H), 7.63–7.59 (m, 1H), 7.56–7.49 (m, 3H), 7.08 (d, J = 8.8 Hz, 2H), 7.02 (d, J = 8.8 Hz, 2H), 6.94 (d, J = 8.8 Hz, 2H), 6.87 (d, J = 8.8 Hz, 2H), 6.55 (d, J = 8.4 Hz, 1H), 6.51 (s, 1H), 6.21 (brs, 1H), 5.59 (d, J = 9.6 Hz, 1H), 5.47 (d, J = 9.6 Hz, 1H), 4.61 (quint, J = 6.4 Hz, 1H), 3.84 (s, 3H), 3.71 (t, J = 6.6 Hz, 2H), 3.60–3.59 (m, 8H), 3.52 (t, J = 5.2 Hz, 2H), 3.41–3.38 (m, 2H), 3.13–3.06 (m, 8H), 2.49 (t, J = 6.4 Hz, 2H), 2.27 (t, J = 6.8 Hz, 2H), 2.15 (t, J = 6.8 Hz, 2H), 1.85 (quint, J = 6.4 Hz, 2H), 1.39 (d, J = 6.0 Hz, 3H), 1.34 (d, J = 6.0 Hz, 3H).

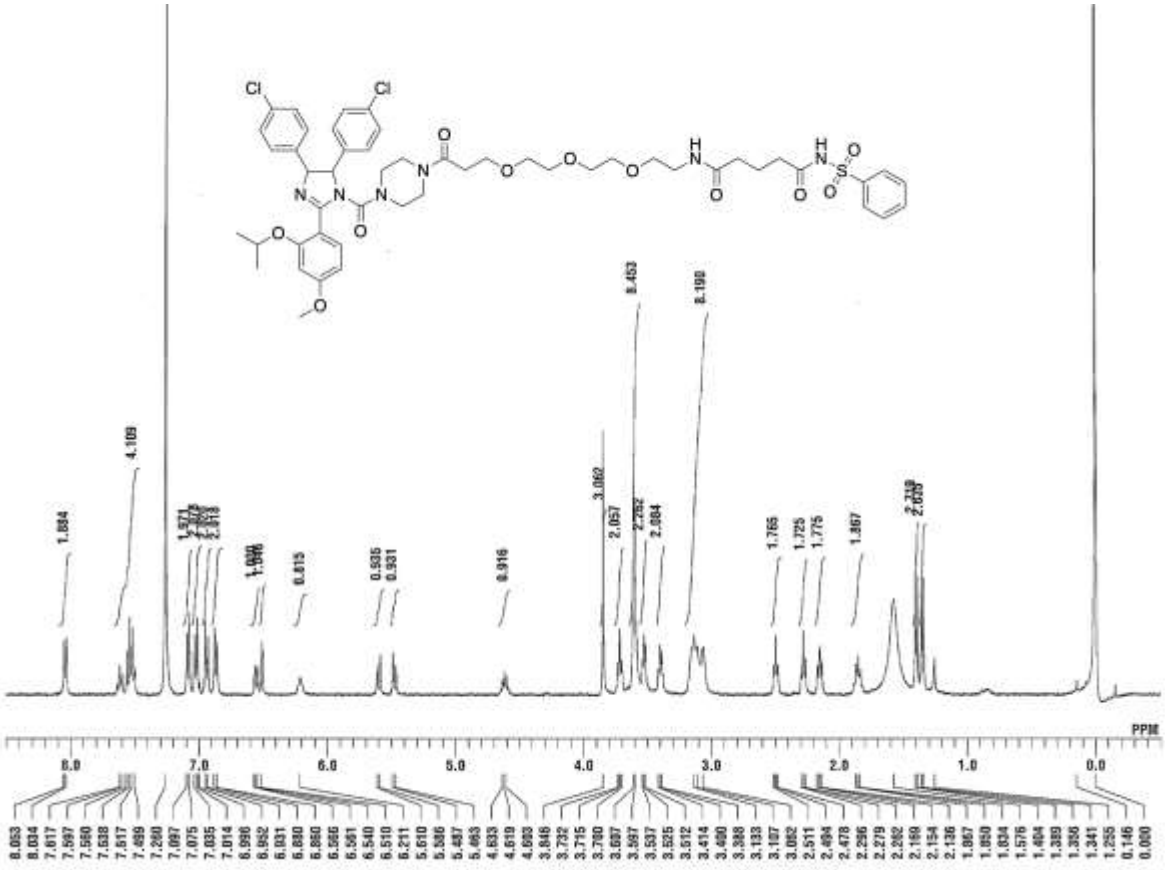

Figure S36 ^{1}H-NMR spectrum of **3b**.

*Covalent inhibitor **3***

To a solution of **3b** (13 mg, 0.0126 mmol) in dry DMF (1 mL) was added DIEA (43 µL, 252 µmol) and iodoacetonitrile (9.1 µL, 126 µmol). The mixture was stirred at room temperature for 24 h in the dark. The solvent was removed under reduced pressure. The residue was

dissolved with $CHCl_3$ (50 mL), washed with 10% citric acid aq. (10 mL) and brine (10 mL), dried over $MgSO_4$ and concentrated under reduced pressure. The resultant was purified by flash chromatography on silica gel (MeOH:$CHCl_3$ = 1:40 to 1:20) to yield **3** (9.1 mg, 0.0085 mmol, 70%) as a colorless oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.98 (d, J = 7.2 Hz, 2H), 7.72 (dd, J = 7.2, 7.2 Hz, 1H), 7.63 (dd, J = 7.2, 7.2 Hz, 2H), 7.53 (d, J = 7.2 Hz, 1H), 7.08 (d, J = 8.4 Hz, 2H), 7.02 (d, J = 8.4 Hz, 2H), 6.94 (d, J = 8.4 Hz, 2H), 6.86 (d, J = 8.4 Hz, 2H), 6.54 (dd, J = 8.4, 2.0 Hz, 1H), 6.50 (d, J = 2.0 Hz, 1H), 6.27 (brs, 1H), 5.59 (d, J = 10 Hz, 1H), 5.47 (d, J = 10 Hz, 1H), 4.76 (s, 2H), 4.61 (quint, J = 6.0 Hz, 1H), 3.85 (s, 3H), 3.72 (t, J = 6.4 Hz, 2H), 3.58–3.56 (m, 8H), 3.50 (t, J = 5.2 Hz, 2H), 3.38 (quint, J = 5.2 Hz, 2H), 3.13–3.07 (m, 8H), 2.76 (t, J = 7.2 Hz, 2H), 2.51 (t, J = 6.4 Hz, 2H), 2.15 (t, J = 7.2 Hz, 2H), 1.90 (quint, J = 6.8 Hz, 2H), 1.39 (d, J = 6.4 Hz, 3H), 1.35 (d, J = 6.4 Hz, 3H).

^{13}C NMR (150 MHz, $CDCl_3$) δ 171.9, 171.7, 169.4, 162.9, 160.3, 157.3, 155.5, 138.1, 136.3, 135.3, 134.9, 133.1, 132.9, 132.0, 129.9, 129.3, 128.5, 128.1, 128.0, 127.8, 114.7, 113.7, 104.5, 100.3, 71.6, 71.0, 70.5, 70.5, 70.4, 70.2, 69.8, 69.2, 67.3, 55.6, 46.2, 45.5, 44.9, 40.8, 39.2, 35.1, 34.7, 33.5, 33.1, 29.7, 22.7, 22.2, 22.1, 20.3

HRMS (ESI) calcd for $[C_{52}H_{61}Cl_2N_7O_{11}S_1Na_1]^+$ $(M+Na)^+$: *m/z* 1084.3419, found 1084.3393.

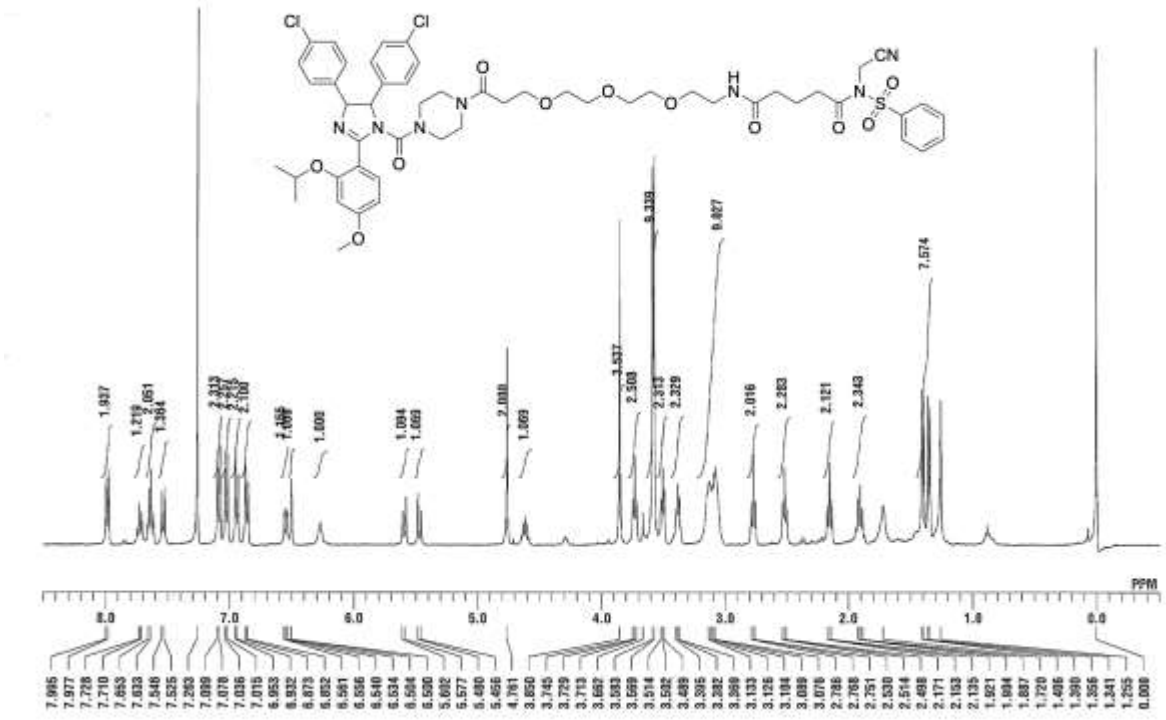

Figure S37 ^{1}H-NMR spectrum of **3**.

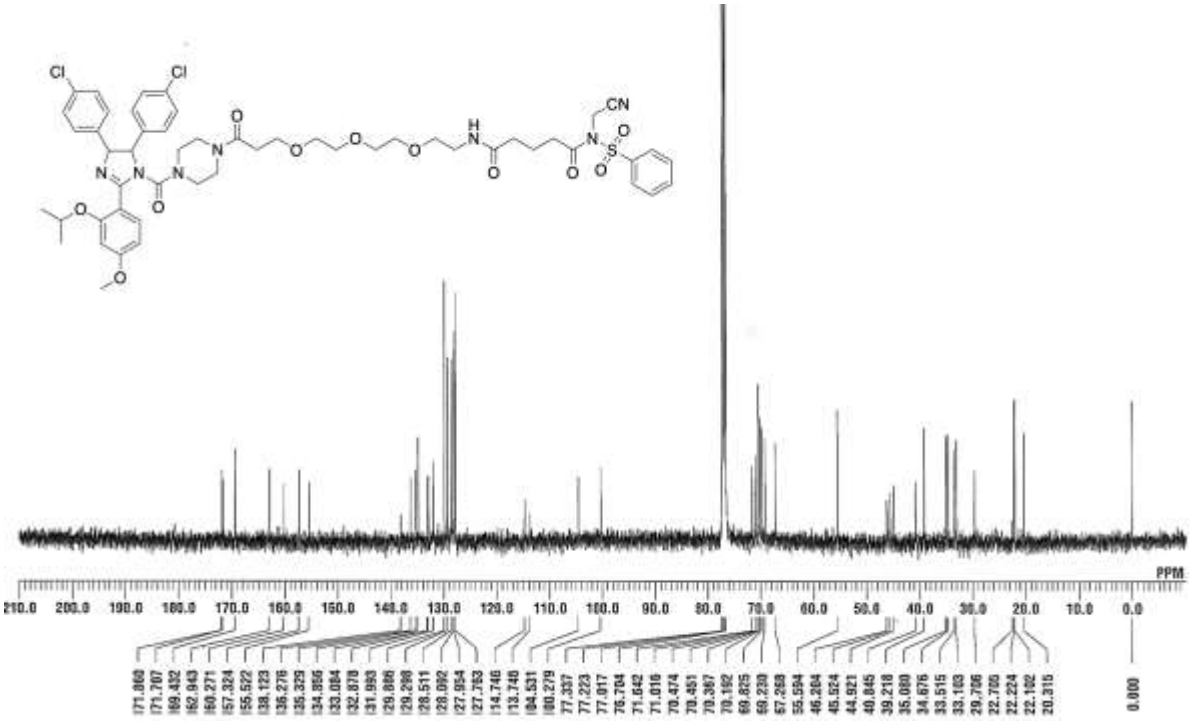

Figure S38 ^{13}C-NMR spectrum of **3**.

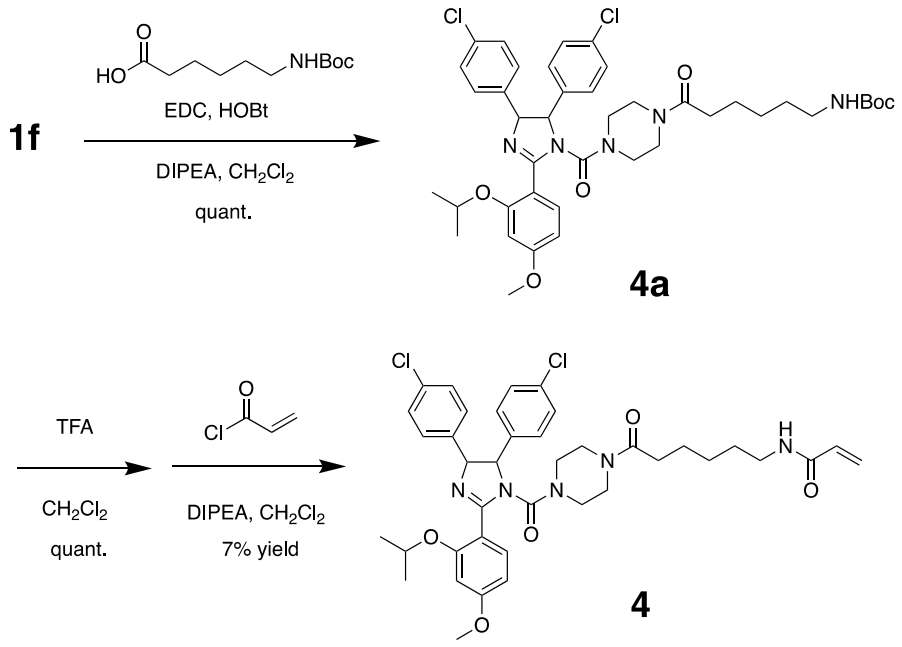

Figure S39 Synthetic scheme of covalent inhibitor **4**.

*Compound **4a***

To a stirred solution of **1f** (5 mg, 0.007 mmol) in dry CH_2Cl_2 (2 mL) was added Boc-6-Ahx-OH (1.9 mg, 0.0084 mmol), EDC (1.6 mg, 0.0084 mmol), 1-hydroxybenzotriazole (HOBt) (1.1 mg, 0.0084 mmol) and DIPEA (6.0 µL, 0.035 mmol). The mixture was stirred at room temperature for 24 h. The solvent was removed under reduced pressure. The residue was dissolved with $CHCl_3$ (50 mL), washed with 10% citric acid aq. (10 mL), sat. $NaHCO_3$ aq. (8 mL) and brine (10 mL), dried over $MgSO_4$ and concentrated under reduced pressure. The resultant was purified by flash chromatography on silica gel (MeOH:$CHCl_3$ = 1:50 to 1:20) to yield **4a** (6 mg, 0.007 mmol, quant.) as a colorless oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.54 (d, J = 8.4 Hz, 1H), 7.09 (d, J = 8.4 Hz, 2H), 7.03 (d, J = 8.4 Hz, 2H), 6.94 (d, J = 8.4 Hz, 2H), 6.86 (d, J = 8.4 Hz, 2H), 6.55 (dd, J = 8.4, 2.0 Hz, 1H), 6.50 (d, J = 2.0 Hz, 1H), 5.58 (d, J = 10 Hz, 1H), 5.46 (d, J = 10 Hz, 1H), 4.61 (quint, J = 6.4

Hz, 1H), 4.53 (brs, 1H), 3.85 (s, 3H), 3.11–2.94 (m, 10H), 2.20 (t, J = 7.2 Hz, 2H), 1.57–1.25 (m, 6H), 1.43 (s, 9H), 1.40 (d, J = 6.4 Hz, 3H), 1.35 (d, J = 6.4 Hz, 3H).

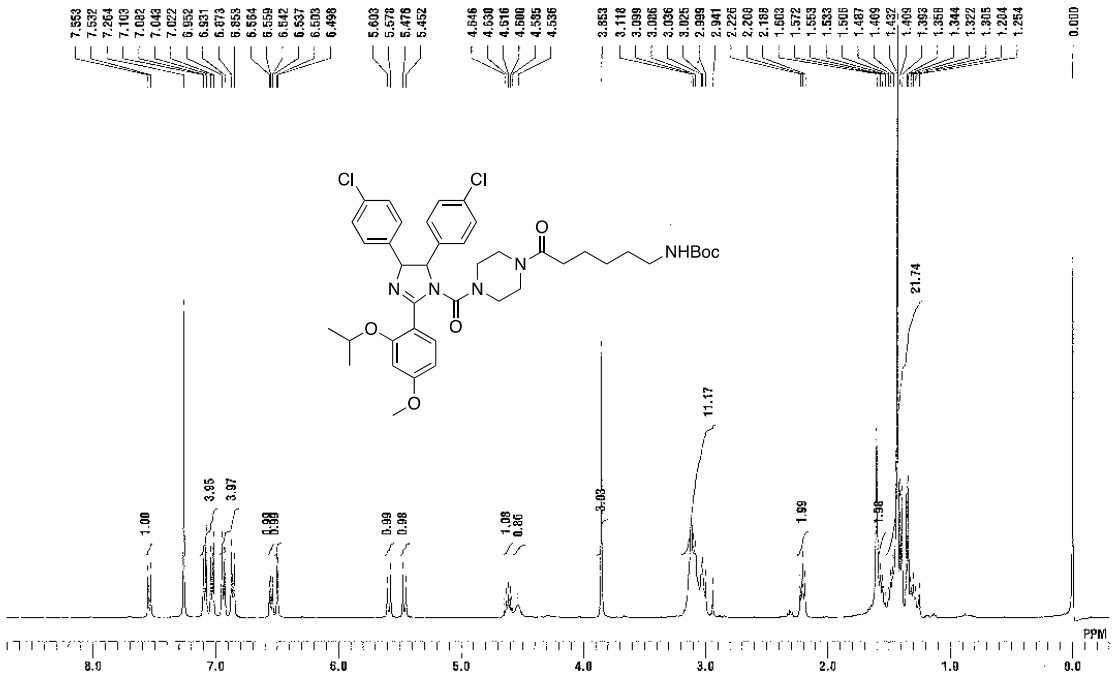

Figure S40 ^{1}H-NMR spectrum of **4a**.

Covalent inhibitor **4**

To a solution of **4a** (6 mg, 0.007 mmol) in CH_2Cl_2 (1 mL) was added TFA (0.5 mL) at room temperature. The reaction solution was allowed to stir for 30 min. TFA was removed by twice co-evaporation with toluene (1 mL). To a stirred solution of this residue in dry CH_2Cl_2 (1 mL) was added acryloyl chloride (1.2 µL, 0.015 mmol) and DIPEA (6.6 µL, 0.038 mmol). The mixture was stirred at room temperature for 1 h. The solvent was removed under reduced pressure. The residue was dissolved with $CHCl_3$ (50 mL), washed with 10% citric acid aq. (10 mL), sat. $NaHCO_3$ aq. (8 mL) and brine (10 mL), dried over $MgSO_4$ and concentrated under reduced pressure. The resultant was purified by RP-HPLC (gradient (A:B) 0 min; 40:60, 40 min; 80:20) to yield **4** (0.4 mg, 0.5 µmol, 7%) as a white solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.69 (d, J = 8.8 Hz, 1H), 7.15 (d, J = 8.4 Hz, 2H), 7.10 (d, J = 8.4 Hz, 2H), 6.96 (d, J = 8.4 Hz, 2H), 6.81 (d, J = 8.4 Hz, 2H), 6.62 (d, J = 8.4 Hz, 1H), 6.54

(s, 1H), 6.26 (m, 1H), 6.07 (m, 1H), 5.85 (m, 1H), 5.75 (m, 1H), 5.63 (m, 1H), 4.68 (m, 1H), 4.29 (m, 1H), 3.89 (s, 3H), 3.33 (q, J = 6.0 Hz, 2H), 3.19–3.07 (m, 8H), 2.22 (t, J = 7.2 Hz, 2H), 1.43 (d, J = 6.0 Hz, 3H), 1.40 (d, J = 6.0 Hz, 3H), 1.33–1.25 (m, 6H).

HRMS (ESI) calcd for $[C_{39}H_{46}Cl_2N_5O_5]^+$ $(M+H)^+$: *m/z* 734.2871, found 734.2866.

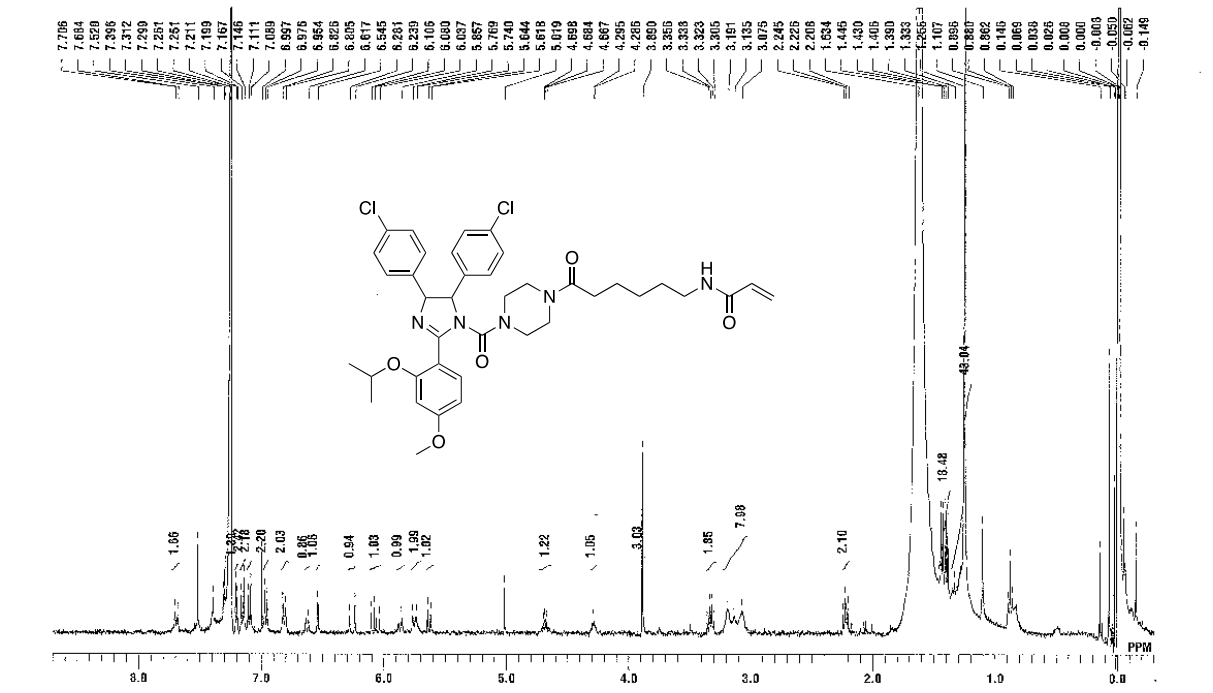

Figure S41 ^{1}H-NMR spectrum of **4**.

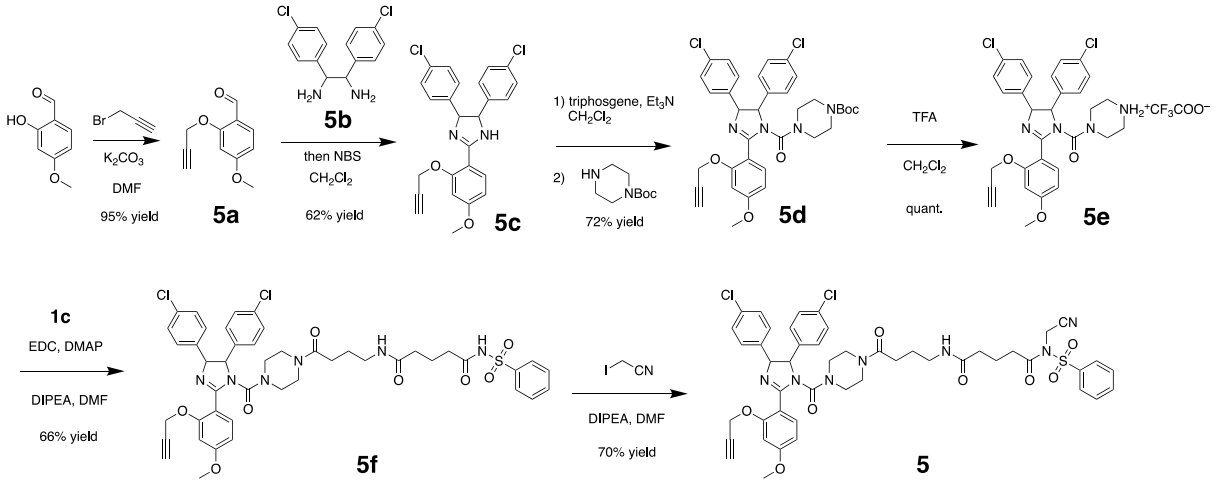

Figure S42 Synthetic scheme of covalent inhibitor **5**.

Compound ***5a***

To a solution of 2-hydroxy-4-methoxybenzaldehyde (1.2 g, 8.2 mmol) and propargyl bromide (1.3 mL, 16 mmol) dissolved in DMF (32 mL) was added K_2CO_3 (3.4 g, 24 mmol). The reaction mixture was stirred at 40 ºC for 2 h and solvent was removed. The resultant was purified by flash chromatography on silica gel (EtOAc:hexane = 1:5) to yield **5a** (1.5 g, 7.8 mmol, 95%) as a white solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 10.30 (s, 1H), 7.84 (d, J = 9.2 Hz, 1H), 6.60 (d, J = 9.2 Hz, 1H), 6.59 (s, 1H), 4.80 (d, J = 2.4 Hz, 2H), 3.88 (s, 3H), 2.58 (d, J = 2.4 Hz, 1H).

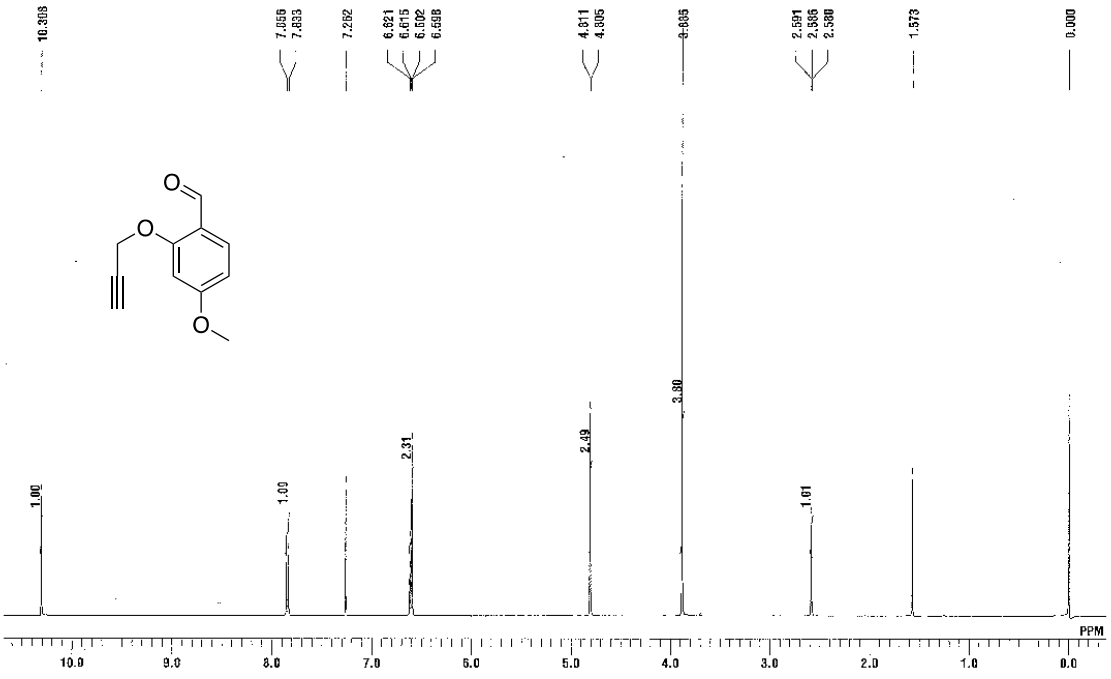

Figure S43 ^{1}H-NMR spectrum of **5a**.

Compound ***5c***

To a solution of **5a** (357 mg, 1.88 mmol) in CH_2Cl_2 (9 mL) was added diamine **5b**[S6] (582 mg, 2.06 mmol). After stirring at room temperature for 2 h, *N*-bromosuccinimide (NBS) (366 mg, 2.06 mmol) was added to the mixture. The reaction mixture was stirred for 12 h. The resulting

mixture was quenched with 10% NaOH aqueous solution and the mixture was extracted with CH_2Cl_2. The extracts were washed with brine and dried over $MgSO_4$, filtered, and concentrated. The resultant was purified by flash chromatography on silica gel (Methanol:CHCl3 = 1:10) to yield **5c** (530 mg, 1.17 mmol, 62%) as a yellowish sticky oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.25 (d, J = 8.8 Hz, 1H), 7.03 (d, J = 8.8 Hz, 4H), 6.91 (d, J = 8.8 Hz, 4H), 6.68–6.64 (m, 2H), 5.35 (m, 2H), 4.80 (d, J = 2.4 Hz, 2H), 3.88 (s, 3H), 2.55 (d, J = 2.4 Hz, 1H).

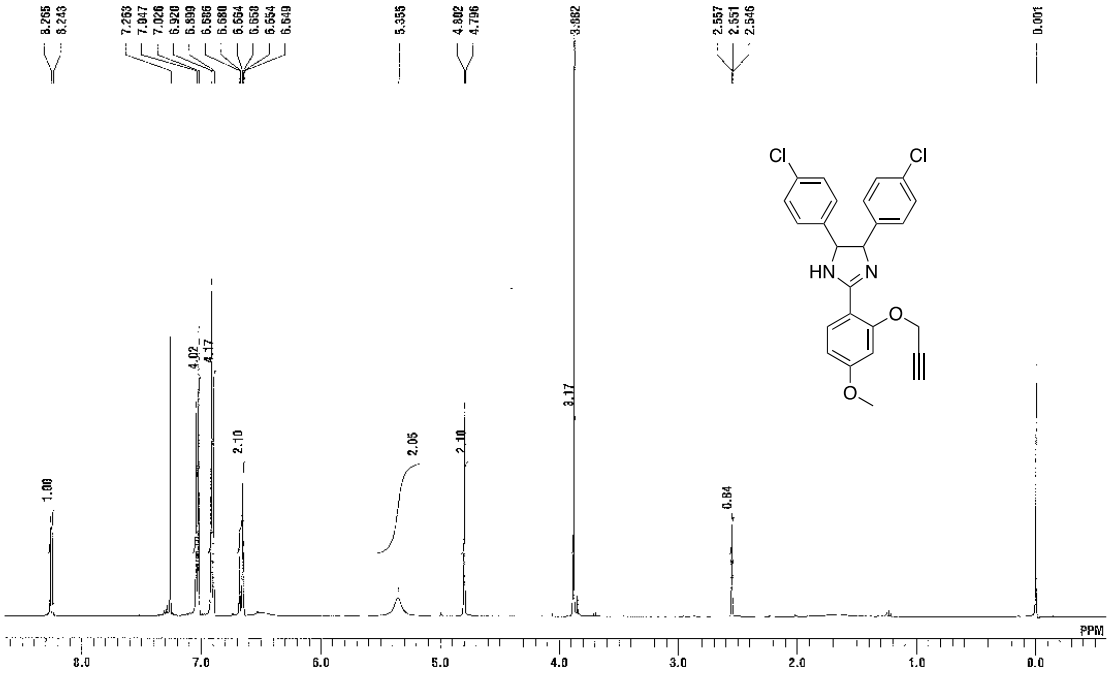

Figure S44 ^{1}H-NMR spectrum of **5c**.

*Compound **5d***

To a solution of **5c** (530 mg, 1.17 mmol) and Et_3N (1.10 mL, 8.21 mmol) dissolved in CH_2Cl_2 (100 mL) at 0 ºC was added a solution of bis(trichloromethyl)carbonate (574 mg, 1.93 mmol) in CH_2Cl_2 (17 mL). The reaction mixture was stirred at 0 ºC for 30 min and solvent was removed. The residue was dissolved in CH_2Cl_2 (100 mL), and a solution of tert-butoxycarbonyl piperazine (3.2 g, 17.5 mmol) in CH_2Cl_2 (17 mL) was added. The

resulting mixture was warmed up to room temperature and stirred for 30 min. The reaction was quenched with 10% citric acid aq. (50 mL), and extracted with CH_2Cl_2. The organic phase was washed with brine (10 mL), dried over $MgSO_4$ and concentrated under reduced pressure. The resultant was purified by flash chromatography on silica gel (EtOAc:hexane = 1:2 to 2:1) to yield **5d** (563 mg, 0.849 mmol, 72%) as a white solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.59 (d, J = 8.8 Hz, 1H), 7.09 (d, J = 8.8 Hz, 2H), 7.04 (d, J = 8.8 Hz, 2H), 6.93 (d, J = 8.8 Hz, 2H), 6.83 (d, J = 8.8 Hz, 2H), 6.69 (d, J = 2.4 Hz, 1H), 6.63 (dd, J = 8.8, 2.4 Hz, 1H), 5.61 (d, J = 9.6 Hz, 1H), 5.48 (d, J = 9.6 Hz, 1H), 4.74 (t, J = 2.0 Hz, 2H), 3.87 (s, 3H), 3.09–2.99 (m, 8H),2.59 (t, J = 2.0 Hz, 1H), 1.41 (s, 9H).

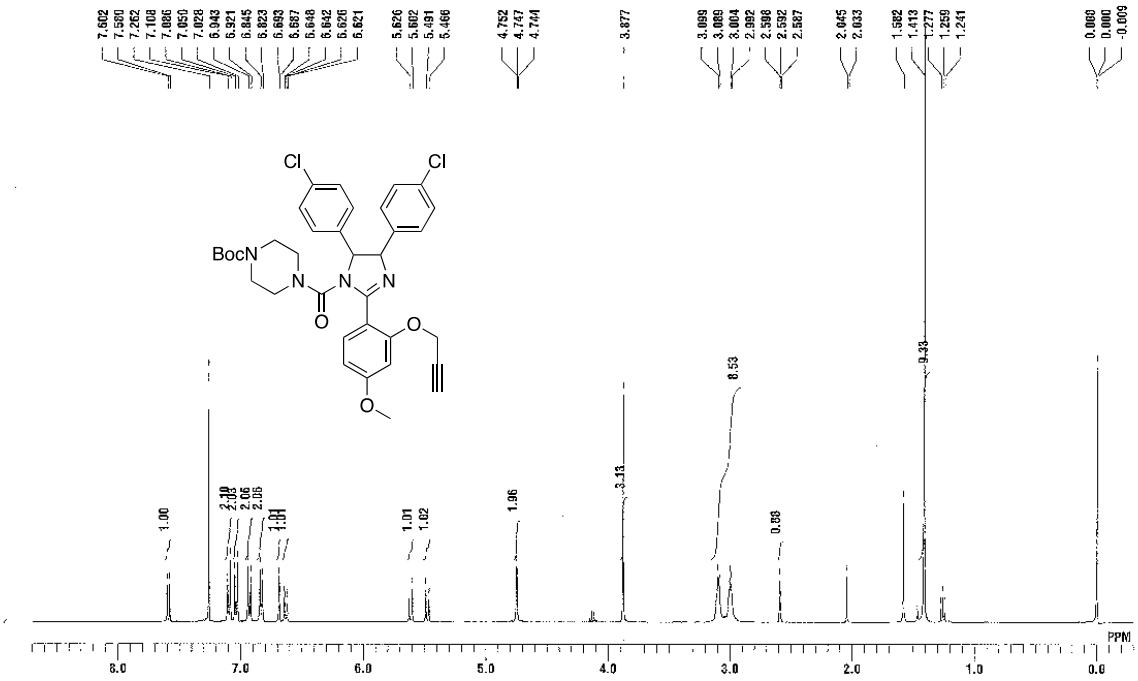

Figure S45 ^{1}H-NMR spectrum of **5d**.

*Compound **5e***

To a solution of **5d** (37 mg, 0.056 mmol) in CH_2Cl_2 (1 mL) was added TFA (0.5 mL) at room temperature. The reaction solution was allowed to stir for 30 min. TFA was removed by twice

co-evaporation with toluene (1 mL) to afford **5e** (38 mg, 0.056 mmol). The product was used in the next step without further purification.

*Compound **5f***

To a stirred solution of this residue and **1c** (0.067 mmol) in dry DMF (2 mL) was added EDC (12 mg, 0.067 mmol), DMAP (8.1 mg, 0.067 mmol) and DIPEA (58 μL, 0.33 mmol). The mixture was stirred at room temperature for 9 h. The solvent was removed under reduced pressure. The residue was dissolved with $CHCl_3$ (50 mL), washed with 10% citric acid aq. (5 mL) and brine (4 mL), dried over $MgSO_4$ and concentrated under reduced pressure. The resultant was purified by flash chromatography on silica gel (MeOH:$CHCl_3$ = 1:50 to 1:10 + 0.5% AcOH) to yield **5f** (33 mg, 0.036 mmol, 66%) as a white solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.03 (d, J = 7.6 Hz, 2H), 7.63–7.61 (m, 2H), 7.51-7.50 (m, 2H), 7.18-7.14 (m, 1H), 7.08 (d, J = 8.8 Hz, 2H), 7.02 (d, J = 8.8 Hz, 2H), 6.91 (d, J = 8.8 Hz, 2H), 6.84 (d, J = 8.8 Hz, 2H), 6.69 (d, J = 2.4 Hz, 1H), 6.63 (dd, J = 8.8, 2.4 Hz, 1H), 5.61 (d, J = 9.6 Hz, 1H), 5.49 (d, J = 9.6 Hz, 1H), 4.75 (d, J = 2.0 Hz, 2H), 3.86 (s, 3H), 3.27–3.03 (m, 10H), 2.64 (t, J = 2.4 Hz, 1H), 2.35–2.17 (m, 6H), 1.86-1.79 (m, 4H).

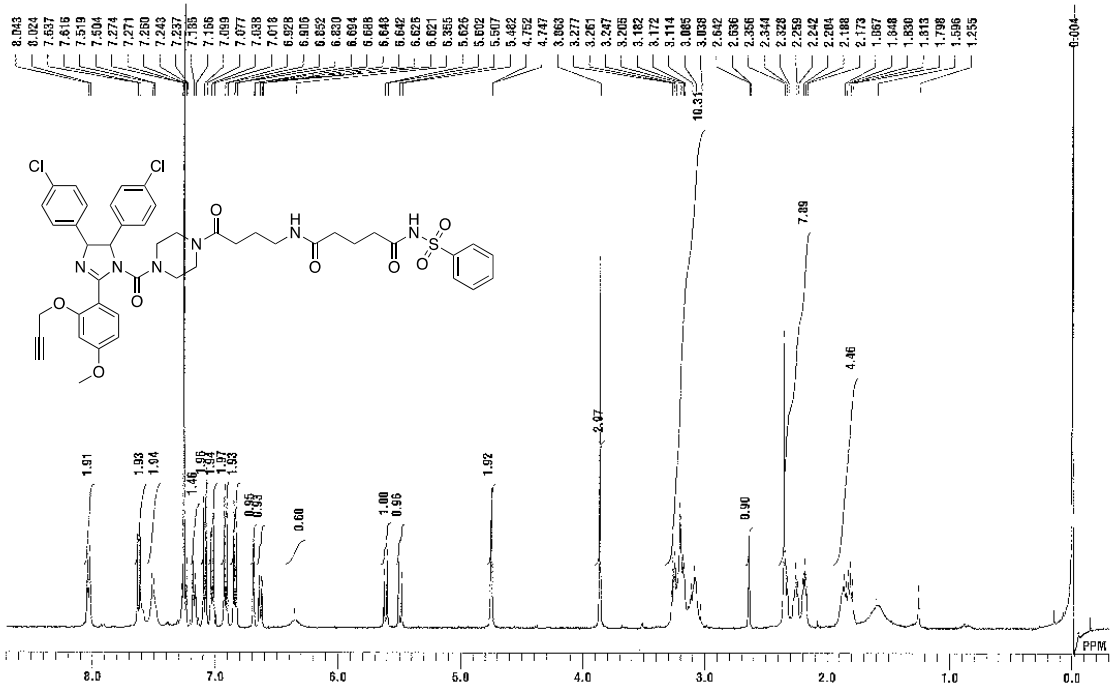

Figure S46 ^{1}H-NMR spectrum of **5f**.

Covalent inhibitor **5**

To a solution of **5f** (33 mg, 0.036 mmol) in dry DMF (1.0 mL) was added DIPEA (62 μL, 0.36 mmol) and iodoacetonitrile (13 μL, 0.18 mmol). The mixture was stirred at room temperature for 15 h in the dark. The solvent was removed under reduced pressure. The residue was dissolved with $CHCl_3$ (50 mL), washed with 10% citric acid aq. (10 mL) and brine (10 mL), dried over $MgSO_4$ and concentrated under reduced pressure. The resultant was purified by flash chromatography on silica gel (MeOH:$CHCl_3$ = 1:40 to 1:20) to yield **5** (23 mg, 0.024 mmol, 70%) as a white solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.98 (d, J = 8.4 Hz, 2H), 7.73 (m, 1H), 7.65-7.60 (m, 3H), 7.09 (d, J = 8.8 Hz, 2H), 7.03 (d, J = 8.8 Hz, 2H), 6.92 (d, J = 8.8 Hz, 2H), 6.84 (d, J = 8.8 Hz, 2H), 6.69 (d, J = 2.0 Hz, 1H), 6.63 (dd, J = 8.8, 2.0 Hz, 1H), 6.01 (m, 1H), 5.60 (d, J = 10.4 Hz, 1H), 5.49 (d, J = 10.4 Hz, 1H), 4.75-4.74 (m, 4H), 3.87 (s, 3H), 3.24–3.04 (m, 10H), 2.75 (t, J = 6.8 Hz, 2H), 2.64 (t, J = 2.4 Hz, 1H), 2.27 (t, J = 6.8 Hz, 2H), 2.12 (t, J = 6.8 Hz, 2H), 1.89 (t, J = 6.8 Hz, 2H), 1.78 (t, J = 6.8 Hz, 2H).

^{13}C NMR (150 MHz, $CDCl_3$) δ 171.9, 171.7, 171.0, 162.9, 160.0, 156.8, 155.6, 138.0, 136.1, 135.1, 134.9, 133.2, 132.9, 131.8, 129.9, 129.2, 128.4, 128.2, 128.0, 127.7, 114.7, 113.2, 105.8, 99.8, 77.8, 76.8, 71.8, 69.2, 56.3, 55.7, 45.9, 45.4, 41.0, 39.3, 35.1, 34.9, 33.1, 30.8, 24.3, 20.3

HRMS (ESI) calcd for $[C_{47}H_{48}Cl_2N_7O_8S_1]^+$ $(M+H)^+$: *m/z* 940.2657, found 940.2655.

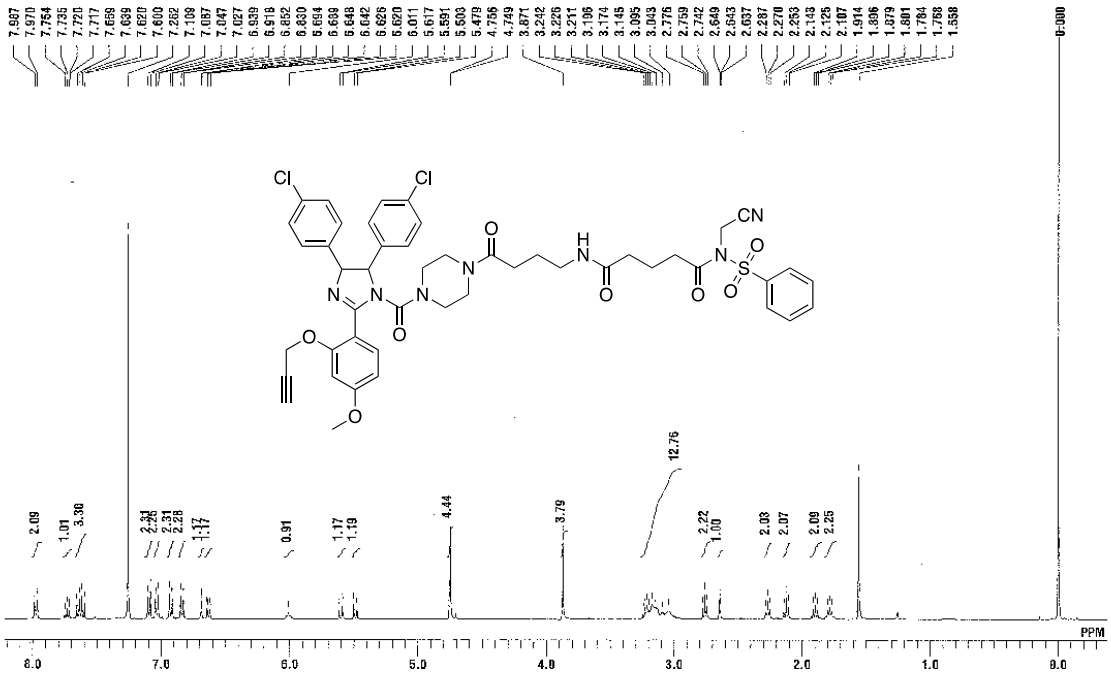

Figure S47 ^{1}H-NMR spectrum of **5**.

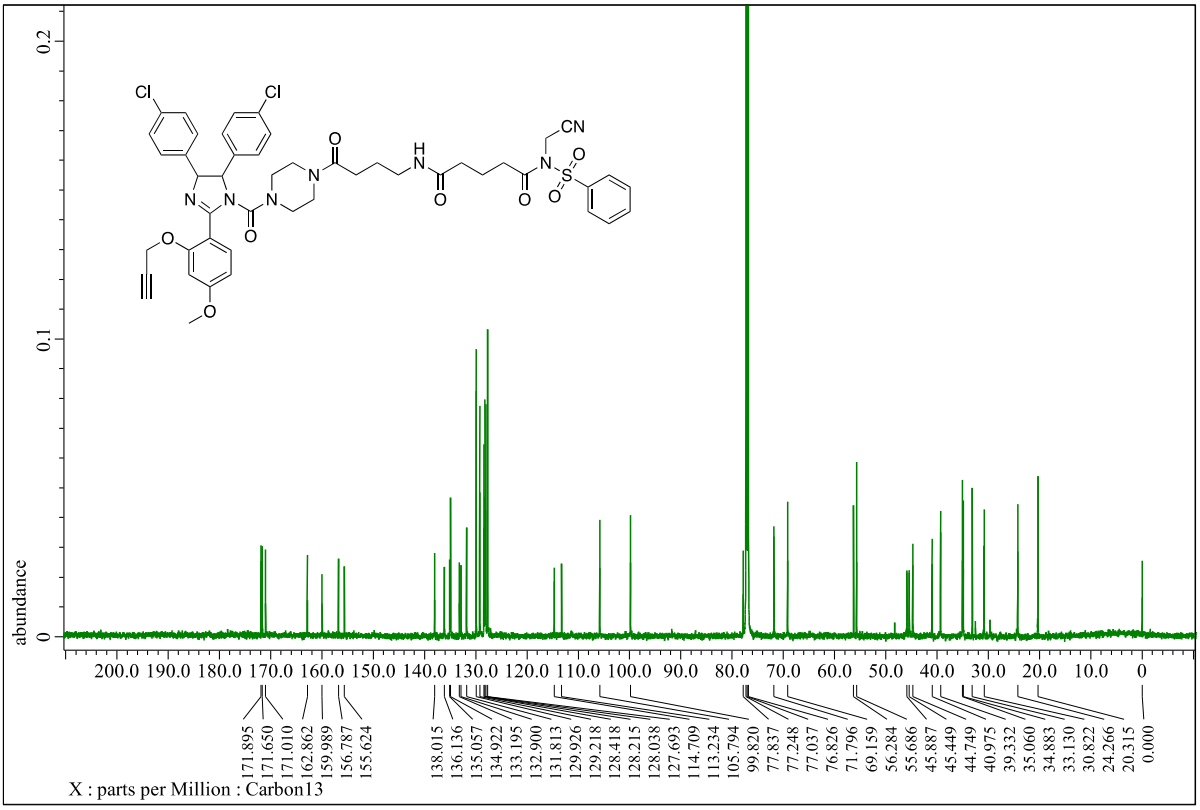

Figure S48 ^{13}C-NMR spectrum of **5**.

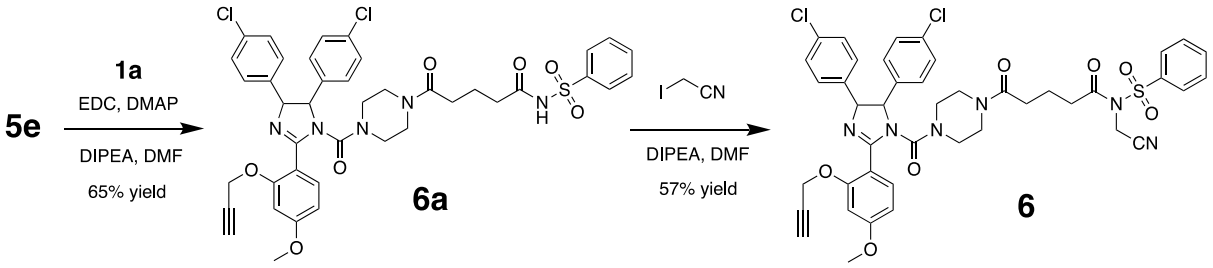

Figure S49 Synthetic scheme of covalent inhibitor **6**.

*Compound **6a***

To a stirred solution of **5e** (21 mg, 0.032 mmol) and **1a** (8.7 mg, 0.032 mmol) in dry DMF (1 mL) was added EDC (10 mg, 0.053 mmol), DMAP (6.0 mg, 0.048 mmol) and DIPEA (45 μL, 0.256 mmol). The mixture was stirred at room temperature for 18 h. The solvent was removed under reduced pressure. The residue was dissolved in $CHCl_3$ (30 mL), washed with 5% citric acid aq. (10 mL x2) and brine (10 mL x2), dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by flash chromatography on silica gel (MeOH:$CHCl_3$ = 1:25) to yield **6a** (17 mg, 0.021 mmol, 65%) as a white solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.02 (d, J = 7.6 Hz, 2H), 7.61 (d, J = 8.0 Hz, 1H), 7.54–7.50 (m, 2H), 7.11 (d, J = 8.0 Hz, 2H), 7.05 (d, J = 8.4 Hz, 2H), 6.93 (d, J = 8.0 Hz, 2H), 6.84 (d, J = 8.4 Hz, 2H), 6.70 (s, 1H), 6.64 (d, J = 8.4 Hz, 1H), 5.62 (d, J = 9.6 Hz, 1H), 5.50 (d, J = 9.6 Hz, 1H), 4.76 (s, 2H), 3.87 (s, 3H), 3.17–3.02 (m, 8H), 2.64 (s, 1H), 2.29–2.23 (m, 4H), 1.82–1.79 (m, 2H).

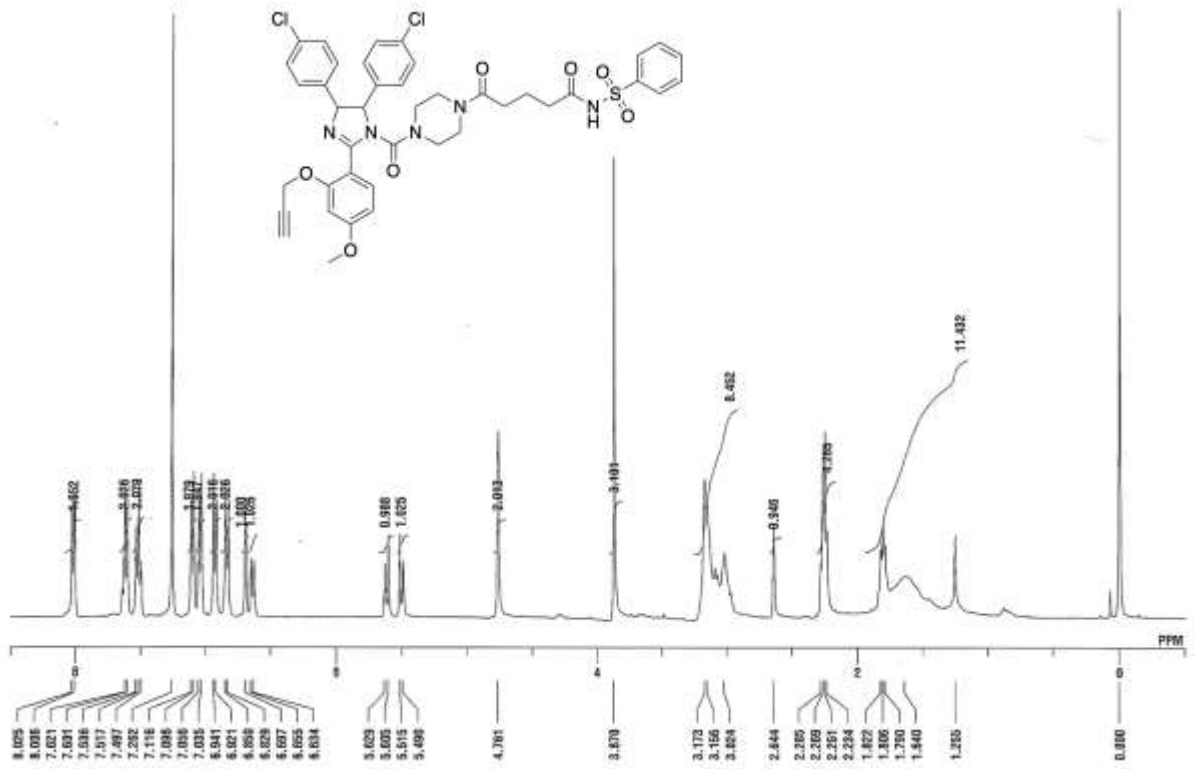

Figure S50 ^{1}H-NMR spectrum of **6a**.

*Compound **6***

To a solution of **6a** (17 mg, 0.021 mmol) in dry DMF (1 mL) was added DIPEA (72 µL, 0.414 mmol) and iodoacetonitrile (15 µL, 0.208 mmol). The mixture was stirred at room temperature for 27 h in the dark. The solvent was removed under reduced pressure. The residue was dissolved in $CHCl_3$ (30 mL), washed with 5% citric acid aq. (10 mL x3) and brine (10 mL x2), dried over $MgSO_4$ and concentrated under reduced pressure. The resultant was purified by flash chromatography on silica gel (MeOH:$CHCl_3$ = 1:30 to 1:10) to yield **6** (10 mg, 0.012 mmol, 57%) as a white solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.96 (d, J = 8.0 Hz, 2H), 7.72 (dd, J = 7.6, 7.2 Hz, 1H), 7.64–7.59 (m, 2H), 7.10 (d, J = 8.0 Hz, 2H), 7.04 (d, J = 8.4 Hz, 2H), 6.93 (d, J = 8.4 Hz, 2H), 6.84 (d, J = 8.4 Hz, 2H), 6.69 (s, 1H), 6.64 (d, J = 8.8 Hz, 1H), 5.61 (d, J = 9.6 Hz, 1H), 5.49 (d, J = 9.6 Hz, 1H), 4.75–4.74 (m, 4H), 3.87 (s, 3H), 3.15–2.96 (m, 8H), 2.76 (t, J = 6.4 Hz, 2H), 2.62 (s, 1H), 2.21 (t, J = 7.2 Hz, 2H), 1.87 (dd, J = 6.4, 7.2 Hz, 2H).

^{13}C NMR (150 MHz, $CDCl_3$) δ 171.8, 170.1, 162.9, 160.0, 156.8, 155.6, 138.0, 136.1, 135.0, 134.9, 133.2, 132.9, 131.8, 129.9, 129.2, 128.4, 128.2, 128.0, 127.7, 114.6, 113.2, 105.8, 99.8, 77.8, 76.7, 71.8, 69.2, 56.2, 55.7, 45.8, 45.4, 44.7, 40.8, 35.0, 33.1, 31.5, 19.8.

HRMS (ESI) calcd for $[C_{43}H_{41}Cl_2N_6O_7S_1]^+$ $(M+H)^+$: *m/z* 855.2129, found 855.2138.

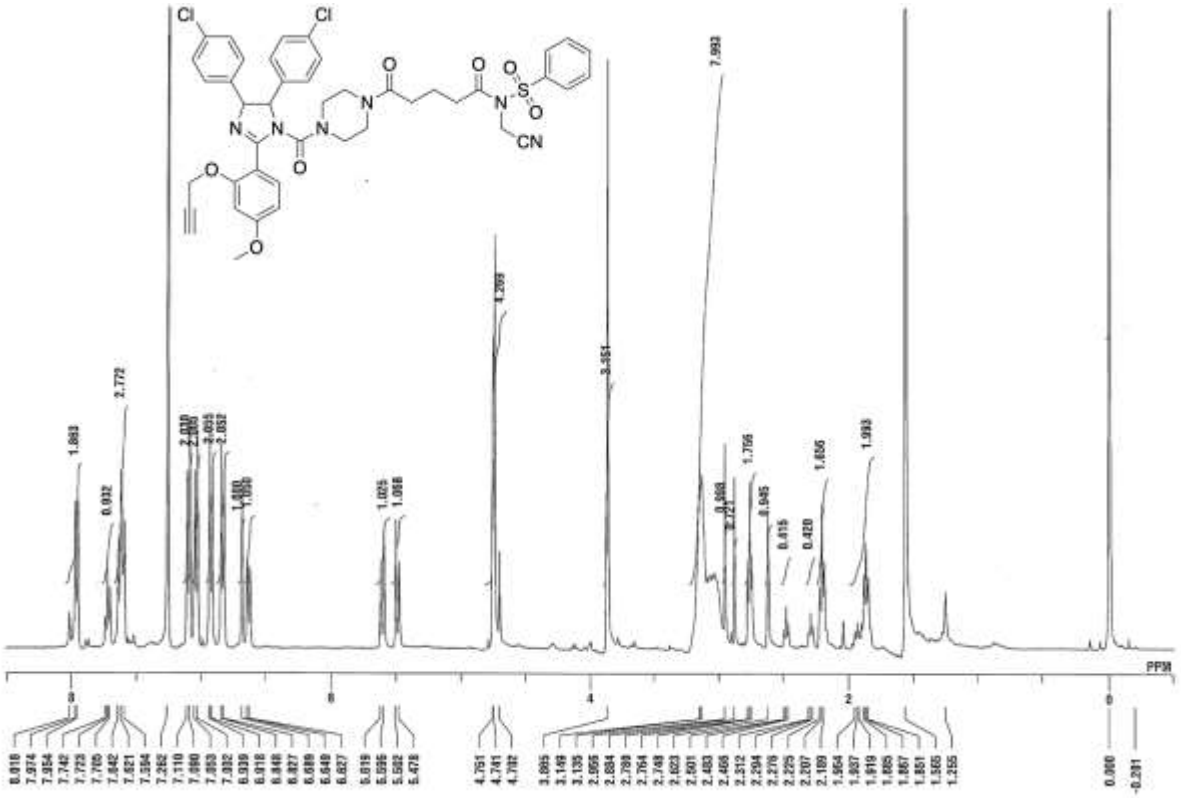

Figure S51 ^{1}H-NMR spectrum of **6**.

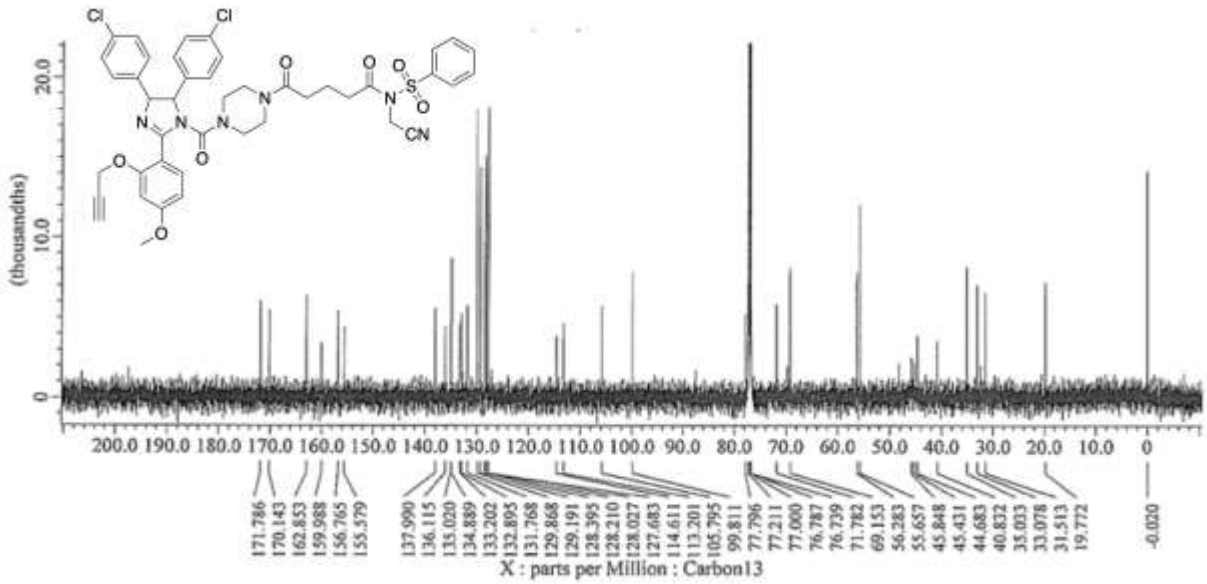

Figure S52 ^{13}C-NMR spectrum of **6**.

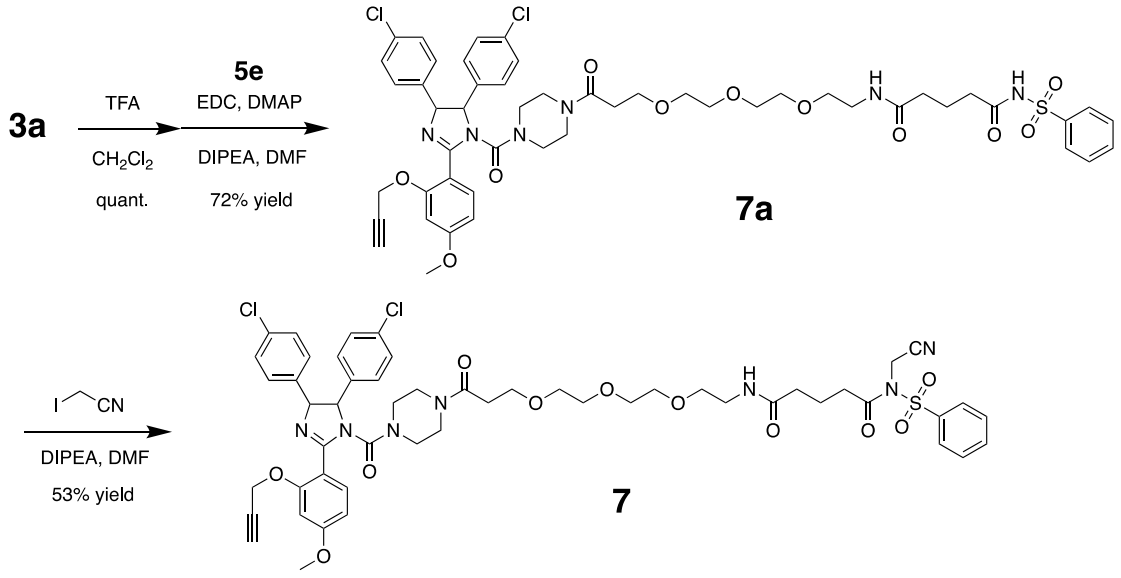

Figure S53 Synthetic scheme of covalent inhibitor **7**.

*Compound **7a***

To a solution of **3a** (19 mg, 0.036 mmol) in CH_2Cl_2 (1 mL) was added TFA (0.5 mL) at room temperature. The reaction solution was allowed to stir for 30 min. TFA was removed by twice co-evaporation with toluene (1 mL). To a stirred solution of this residue in dry DMF (1 mL) was added **5e** (24 mg, 0.036 mmol), EDC (11 mg, 0.055 mmol), DMAP (7 mg, 0.058 mmol)

and DIPEA (50 μL, 0.287 mmol). The mixture was stirred at room temperature for 19 h. The solvent was removed under reduced pressure. The residue was dissolved in $CHCl_3$ (30 mL), washed with 5% citric acid aq. (10 mL x3) and brine (10 mL x2), dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by flash chromatography on silica gel (MeOH:$CHCl_3$ = 1:25 to 1:10) to yield **7a** (26 mg, 0.026 mmol, 72%) as a white solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.04 (d, J = 7.2 Hz, 2H), 7.62 (t, J = 8.4 Hz, 2H), 7.51 (t, J = 7.6 Hz, 2H), 7.10 (d, J = 8.8 Hz, 2H), 7.04 (d, J = 8.8 Hz, 2H), 6.93 (d, J = 8.8 Hz, 2H), 6.85 (d, J = 8.4 Hz, 2H), 6.69 (s, 1H), 6.64 (dd, J = 2, 6.4 Hz, 1H), 6.29 (brs, 1H), 5.62 (d, J = 9.6 Hz, 1H), 5.50 (d, J = 10.4 Hz, 1H), 4.75 (s, 2H), 3.87 (s, 3H), 3.71 (t, J = 6.8 Hz, 2H), 3.60 (m, 8H) 3.52 (t, J = 4.8 Hz, 2H), 3.40 (q, J = 4.4 Hz, 2H), 3.17-3.10 (m, 8H), 2.67 (s 1H), 2.49 (t, J = 6.4, 2H), 2.27 (t, J = 6.8 Hz, 2H), 2.16 (t, J = 6.8 Hz, 2H), 1.85 (quin, J = 6.8 Hz, 2H).

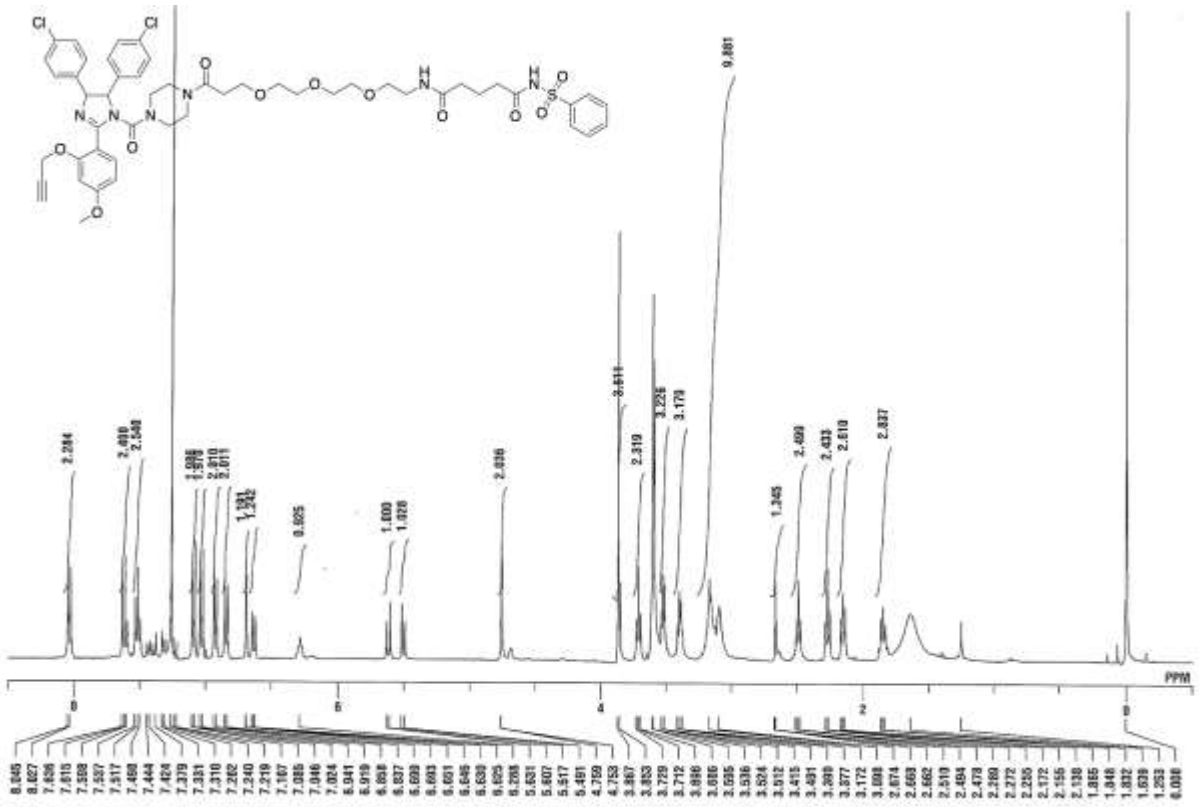

Figure S54 ^{1}H-NMR spectrum of **7a**.

Compound ***7***

To a solution of **7a** (26 mg, 0.026 mmol) in dry DMF (1 mL) was added DIPEA (90 µL, 0.514 mmol) and iodoacetonitrile (19 µL, 0.257 mmol). The mixture was stirred at room temperature for 40 h in the dark. The solvent was removed under reduced pressure. The residue was dissolved in $CHCl_3$ (30 mL), washed with 5% citric acid aq. (10 mL x3) and brine (10 mL), dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by flash chromatography on silica gel (MeOH:$CHCl_3$ = 1:30 to 1:10) to yield **7** (14 mg, 0.014 mmol, 53%) as a white solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.99 (d, J = 8.0 Hz, 2H), 7.73 (t, J = 7.6 Hz, 1H), 6.63 (q, J = 6.8 Hz, 3H), 7.10 (d, J = 8.4 Hz, 2H), 7.04 (d, J = 8.8 Hz, 2H), 6.93 (d, J = 8.4Hz, 2H), 6.84 (d, J = 8.8Hz, 2H), 6.69 (s, 1H), 6.64 (dd, J = 2, 6.4 Hz, 1H), 6.46 (brs, 1H), 5.61 (d, J = 10 Hz, 1H), 5.50 (d, J = 10.4 Hz, 1H), 4.76 (s, 4H), 3.87 (s, 3H), 3.73 (t, J = 6.4 Hz, 2H), 3.58 (s, 8H), 3.51 (t, J = 5.2, 2H), 3.38 (q, J = 5.2 Hz, 2H), 3.16-3.11 (m, 8H), 2.77 (t, J = 6.8 Hz, 2H), 2.65 (s, 1H), 2.52 (t, J = 6.0 Hz, 2H), 2.16 (t, J = 7.6 Hz, 2H), 1.91 (quint, J = 7.2 Hz, 2H).

^{13}C NMR (150 MHz, $CDCl_3$) δ 171.9, 171.7, 169.4, 162.8, 160.0, 156.8, 155.6, 138.0, 136.1, 135.0, 134.8, 133.2, 132.9, 131.8, 129.9, 129.2, 128.4, 128.2, 128.0, 127.7, 114.8, 113.2, 105.8, 99.8, 77.8, 71.7, 70.4, 70.3, 70.1, 69.8, 69.1, 67.1, 56.3, 55.6, 46.0, 45.3, 44.9, 40.9, 39.2, 35.0, 34.6, 33.4, 33.1, 20.3.

HRMS (ESI) calcd for $[C_{52}H_{57}Cl_2N_7O_{11}S_1Na_1]^+$ $(M+Na)^+$: *m/z* 1080.3106, found 1080.3105.

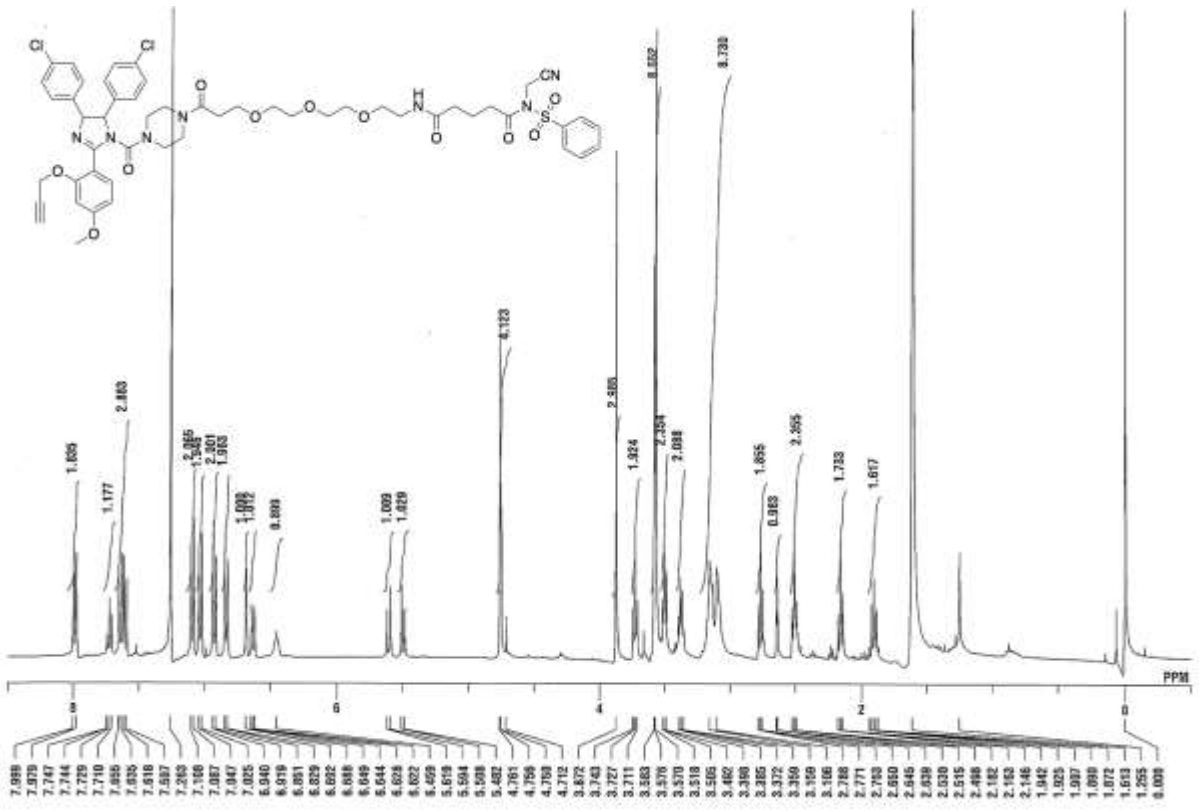

Figure S55 ^{1}H-NMR spectrum of **7**.

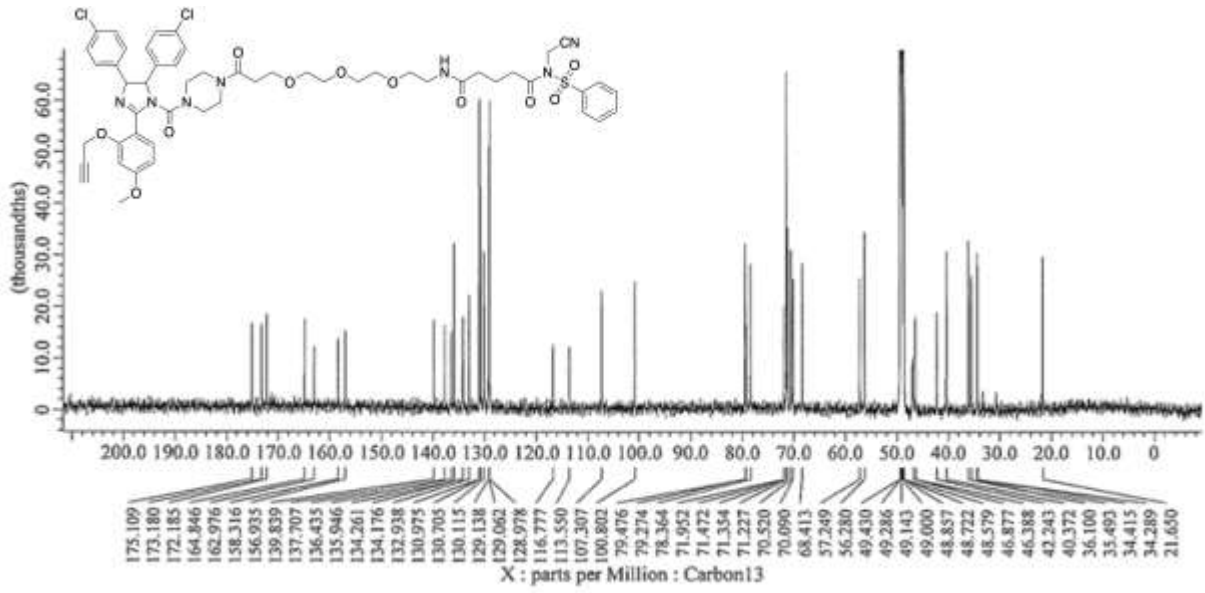

Figure S56 ^{13}C-NMR spectrum of **7**.

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