Supporting Information

Exploring Ligand-directed NASA-based Acylation Chemistry for Potential Targeted Degrader Development

Mingxing Teng,[1,2,†] Jie Jiang,[1,2,†] Scott B. Ficarro,[1,3,4,†] Hyuk-Soo Seo,[1,2] Jae Hyun Bae,[1] Katherine A. Donovan,[1,2] Eric S. Fischer,[1,2] Tinghu Zhang,[5] Sirano Dhe-Paganon,[1,2] Jarrod A. Marto,[1,3,4,] Nathanael S. Gray[5,*]*

[1]Department of Cancer Biology, Dana-Farber Cancer Institute, Boston, MA 02115, USA

[2]Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115, USA

[3]Department of Oncologic Pathology and Blais Proteomics Center, Dana-Farber Cancer Institute, Boston, MA 02115, USA

[4]Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA

[5]Department of Chemical and Systems Biology, ChEM-H, Stanford Cancer Institute, School of Medicine, Stanford University, Stanford, CA 94305

*Correspondence: Jarrod_Marto@dfci.harvard.edu, nsgray01@stanford.edu

†These authors contributed equally

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Table S1. Kinases containing a lysine residue at the same position as Lys89 of CDK2.

Kinase	Residue
CDK1	K89
CDK2	K89
CDK3	K89
CDK5	K89
MAPK1 (ERK2)	K114
MAPK3 (ERK1)	K131
MAPK12 (ERK6)	K120
MAPK13	K116
NLK	K228
SRPK1	K174
SRPK2	K175
SRPK3	K173
NEK1	K90
NEK5	K90
PDPK1	K169
PDPK2P	K142
AURKB	K164
AURKC	K130
VRK1	K140
VRK2	K130
IKBKB	K106
MAP2K6 (MKK6)	K138
MAP2K7 (MKK7)	K221
ANKK1 (PKK2)	K108
BMPR2	K289
RAF1	K431
NRTK2 (Trk B)	K643
NRTK3 (Trk C)	K627
SYK	K458
ZAP70	K424

In vitro kinase assays

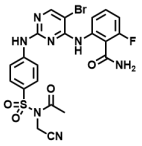
Z'-LYTE assays were conducted for CDK1/cyclin B, CDK2/cyclin A, & CDK5/p25 as performed in the commercial assay service by Invitrogen Technologies in a 10-point dose response using K_m ATP concentrations.

Adapta assays were conducted for CDK4/cyclin D1 & CDK6/cyclin D1 as performed in the commercial assay service by Invitrogen Technologies in a 10-point dose response using 10 mM ATP concentrations.

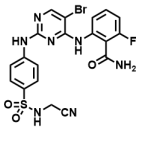
Adapta assays were conducted for CDK7/cyclin H/MNAT1 & CDK9/cyclin T1 as performed in the commercial assay service by Invitrogen Technologies in a 10-point dose response using K_m ATP concentrations.

All IC_{50}s were tested in duplicate.

Table S2. Chemical structures of TMX compounds and their CDKs inhibitory activities.



TMX-3063



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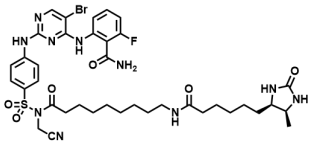
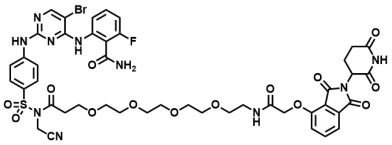
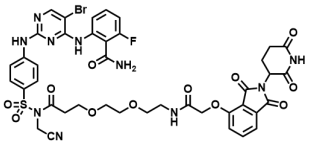


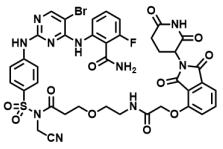
TMX-3052



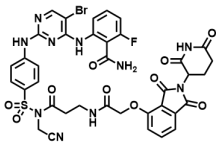
TMX-3054



TMX-3166



TMX-3164



TMX-3185

Compound	TMX-3063	TMX-3066	TMX-3052	TMX-3054	TMX-3166	TMX-3164	TMX-3185
CDK1/cyclin B IC_{50} (nM)	3.8±1.0	2.2±0.2	27.8±4.5	9.7±0.8	8.5±1.0	34.5±3.0	48.6±2.2
CDK2/cyclin A IC_{50} (nM)	2.2±0.1	1.7±0.1	22.1±1.0	3.5±0.5	3.1±0.3	8.2±0.6	16.9±1.0
CDK3/cyclin E1 IC_{50} (nM)	11.3±1.4	7.7±0.7	ND[a]	14.5±3.3	13.4±0.9	34.5±3.4	47.1±8.4
CDK5/p25 IC_{50} (nM)	1.6±0.2	0.8±0.1	6.8±0.4	2.3±0.2	10.3±0.5	18.3±0.9	30.0±3.5
CDK4/cyclin D1 IC_{50} (nM)	>370	914.0±387.4	ND	2040.0±4446.0	ND	ND	ND
CDK6/cyclin D1 IC_{50} (nM)	>10000	113.0±28.3	ND	2830.0±794.3	ND	ND	ND
CDK7/cyclin H IC_{50} (nM)	69.7±15.5	43.8±5.5	ND	211.0±42.9	ND	ND	ND
CDK9/cyclin T1 IC_{50} (nM)	19.2±5.1	14.2±1.7	ND	65.3±11.1	ND	ND	ND

[a] Not determined.

Cell culture

OVCAR8 (female) cells were cultured in DMEM media (Life technologies, cat# 11995073) containing 10% fetal bovine serum (Life technologies, cat# 10437028) and 1% Penicillin/Streptomycin (Life technologies, cat# 10378016). All the cells were cultured at 37 °C in 5% CO_2 humidified air and tested for mycoplasma-negative (Lonza, LT07-318).

Immunoblotting analysis

Cells were lysed in RIPA buffer (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) (Sigma, cat# R0278) with protease inhibitor (Sigma, cat# 11836153001)/phosphatase (Sigma, cat# 04906837001) inhibitor. The protein concentrations were measured by BCA analysis (Thermo Fisher Scientific, cat# PI23225). Equal amounts of protein were resolved by 4-12% Tris-Base gels (Life Technologies, cat# NW04125BOX), and then transferred to the Immuno-Blot PVDF membrane (BioRad, cat# 1620177). Proteins were probed

with appropriate primary antibodies at 4 °C overnight and then with IRDye®800-labeled goat anti-rabbit IgG (LICOR Biosciences, cat# 926-32211), IRDye®800-labeled goat anti-mouse IgG (LICOR Biosciences, cat# 926-32210) or IRDye 680RD goat anti-Mouse IgG (LICOR Biosciences, cat# 926-68070) secondary antibodies at room temperature for 1 h. The membranes were detected on Odyssey CLx system.

Antibodies used in this study include anti-following proteins: CDK1 (Abcam, cat# ab131450, 1:1000), CDK2 (Cell Signaling Technology, cat# 2546S, 1:1000), CDK5 (Cell Signaling Technology, cat# 12134S, 1:1000), and β-Actin (Cell Signaling Technology, cat# 3700, 1:1000).

Cellular target engagement assay

After 4 h treatment, cells were pelleted, washed with PBS three times and lysed with IP lysis buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40 and 5% glycerol) (Thermo Fisher Scientific, Cat# 87788) containing protease/phosphatase inhibitor cocktail (Roche). The protein concentrations were measured by BCA analysis (Pierce). Cell lysates were incubated with 1 µM TMX-3052 (desthiobiotinylated probe) at 4°C overnight and incubated for another 3 h at room temperature. Lysates with probe were then incubated with streptavidin beads (Thermo Fisher, Cat# 20349) for 2 h at 4°C. The protein-probe complexes on the beads were then subjected to immunoblotting.

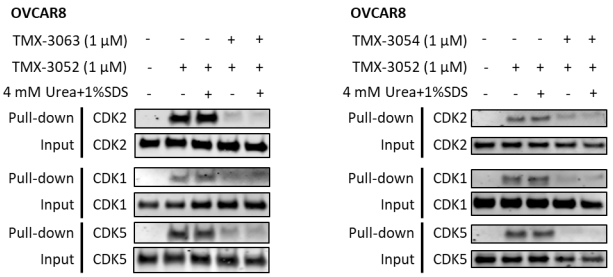


Figure S1. TMX-3063 and TMX-3054 labels CDK1, CDK2, and CDK5 in cells, separately. OVCAR8 cells were pre-treated with either DMSO or TMX-3063 (or TMX-3054) (1 µM) for 4 h followed by cell lysis, then the cell lysates were incubated with TMX-3052 (1 µM) for 16 h. 4 mM Urea+1%SDS was used to denature the protein to eliminate the reversible binding.

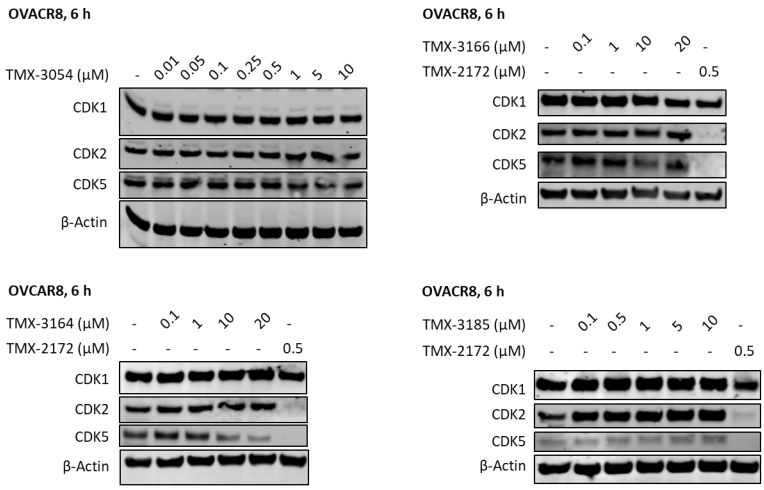


Figure S2. Immunoblot analysis of CDK1, CDK2, and CDK5 treated with the indicated concentration of TMX compounds for 6 h in OVCAR8 cells.

CDK2-HiBit degradation quantitation assay

The sequences of the ssODN donor template and guide RNA were obtain from Promega (Promega, Madison, WI). 5 µL of 100 µM of Alt-R CRISPR-Cas9 crRNA and 5 µL of 100 µM of Alt-R CRISPR-Cas9 tracrRNA were mixed by incubation at 95 °C for 5 min and cooling down by 2°C/min to 25 °C to generate duplex sgRNA. Single-stranded Ultramer DNA Oligos (IDT) were used as the ssODN donor templates. Ribonucleoprotein complexes with recombinant S.p. Cas9 Nuclease V3 (IDT) were assembled by incubating 80 pmol of Cas9 and 100 pmol of gRNA for 10 min at room temperature. A total of 10^6 HEK293T cells were resuspended in 100 µL of 4D Nucleofector Solution SE, and ribonucleoprotein complexes along with 1 µg donor template were electroporated into cells with the 4D Nucleofector System (Lonza, Basel, Switzerland). Immediately following electroporation, cells were incubated at room temperature for 10 min before transferring to a 12-well plate for culturing. 72 h after electroporation, edited pools were analysed for HiBiT insertion using the Nano-Glo HiBiT Lytic Detection System (Promega, Madison, WI) and for measuring luminescence. Clonal populations of edited cells were obtained by sorting live singlets into 96-well plates. Clones were expanded and screened by luminescence. The luminescence positive single clone cells were plated at the density of 20000 cells/well into 96-well plate. The following day, the cells were treated with the compounds for 6 h. Then the Nano-Glo Lytic Reagent was added to all wells, and luminescence was measured.

NanoBRET live-cell ternary complex formation assay

HEK293T cells were cultured overnight and transfected with Lipofectamine 3000 (Thermo Fisher Scientific) and 2 µg of HaloTag-CRBN plasmid and 0.02 µg of NanoBRET CDK2 plasmid in 6-well plates. 24 h later, transfected cells were replated at the density of 20000 cells/well into white 96-well tissue culture plates in the presence or absence of HaloTag NanoBRET 618 Ligand (Promega) in assay medium (Opti-MEM I Reduced Serum Medium, no phenol red + 4% FBS). The following day, the cells were treated with the compounds for 3 h. Then NanoBRET™ Nano-Glo® Substrate (Promega) were added. Donor emission (460 nm) and acceptor emission (618 nm) were measured within 10 minutes of substrate addition using CLARIOstar (BMG Labtech, Offenburg, Germany). Background-subtracted NanoBRET ratios expressed in milliBRET units were calculated by multiplying NanoBRET ratios by 1000, and fold increase in BRET was calculated by normalizing mBRET ratios to the average mBRET ratios for DMSO controls.

Protein expression, purification, and co-crystallization

Residues 1-298 of human CDK2 were inserted into pGEX6P (N-terminal, cleavable GST fusion) with a final plasmid sequence described in genbank; Residues 171-432 of human CCNA2 were inserted into pGEX6P (N-terminal, cleavable GST fusion) with a final plasmid sequence described in genbank. Plasmids of GST-CDK2, bicistronically expressing CAK1, and GST-CCNA2 were transformed into Tuner (DE3) cells (Novagen), expressed in TB media supplemented with 100 µg/mL Ampicilin by adding ITPG to 0.2 mM at OD ~0.4, incubated for 16 hours at 18°C. Cells were collected by centrifugation and stored at -80°C. Cell pellets of GST-CDK2/CAK1 and GST-CCNA2 were resuspended together in buffer A (50 mM sodium HEPES, pH 7.5, 200 mM NaCl, 5% glycerol,

and 7 mM BME), lysed by microfluidizer (Microfluidics), and the resulting lysate was centrifuged at 16,000 g for 30 min. ~5 mL Glutathione beads (Cytiva) were mixed with lysate supernatant for 90 min, washed with buffer A, and transferred to an empty column. ~5 mL HRV3C protease solution prepared in buffer A, supplemented with 100 mM $MgCl_2$ and 0.01% Triton X-100, was added to the washed column for overnight on-column cleavage at 4°C. The eluted sample was further purified by size exclusion chromatography using a Superdex 200 16/600 column in buffer B (20 mM HEPES, pH 7.5, 200 mM NaCl, 5% glycerol, 1 mM DTT, and 0.5 mM TCEP). CDK2/CCNA2 complex containing fractions were pooled, concentrated to ~40 mg/mL, and stored at -80°C. Using Formulatrix NT8 and RockImager and ArtRobbins Phoenix liquid handlers, a 100 nL sample of 300 µM CDK2/CCNA2 complex was dispensed in an equal volume of 1.5M ammonium citrate (pH 6.0) and incubated by sitting-drop vapor diffusion at 20°C for seven days. Diffraction data were collected at beamline 24ID-E of the NE-CAT at the Advanced Photon Source (Argonne National Laboratory). Data sets were integrated and scaled using XDS.

Mass spectrometry analysis

Recombinant human CDK2 was incubated with DMSO or the indicated compound at room temperature and then analysed by ESI-MS using an autosampler and HPLC (Shimadzu, Marlborough, MA) interfaced to an LTQ ion trap mass spectrometer (ThermoFisher Scientific, San Jose, CA) that acquired full scan mass spectra (m/z 300-2000) in profile mode. Mass spectra were deconvoluted using MagTran version 1.03 b2[1].

To determine the site of modification, proteins were denatured with 0.1% rapigest (Waters, Milford, MA), reduced (10 mM DTT, 56 °C, 30 minutes), alkylated (22.5 mM iodoacetamide, 30 min, room temperature, protected from light), and digested with trypsin overnight at 37 °C. Rapigest was cleaved by adding trifluoroacetic acid to a final concentration of 1% and incubating at 37 °C for 30 minutes, and pelleted by centrifugation at 14k g for 10 minutes at 4 °C. Peptides in the supernatant were desalted by C18, dried by vacuum centrifugation, reconstituted in 1% formic acid, 50% acetonitrile with 100 mM ammonium acetate and analysed by CE-MS using a ZipChip Autosampler and CE instrument (908 devices, Boston, MA) interfaced to a QExactive HF mass spectrometer (ThermoFisher Scientific). After peptide injection, CE was performed at 500 V/cm using an HR chip (22 cm separation channel) with a background electrolyte composed of 1% formic acid in 50% acetonitrile. Pressure assist was utilized and started at 1 minute. The mass spectrometer was operated in data dependent mode and subjected the 5 most abundant ions in each MS scan (60k resolution, 1E6 target, lock mass enabled) to MS/MS (15k resolution, 2E5 target, 100 ms max inject time). Dynamic exclusion was enabled with a repeat count of 1 and an exclusion time of 6 seconds. Raw data was extracted to .mgf using mulitplierz scripts[2] and searched against a forward-reverse human NCBI refseq database using Mascot version 2.6.2. Precursor mass tolerance was set to 10 ppm and product ion tolerance was 25 mmu. MS/MS spectra were analyzed using mzStudio software[3].

References

[1] Zhang, Z.; Marshall, A. G., A universal algorithm for fast and automated charge state deconvolution of electrospray mass-to-charge ratio spectra. *J. Am. Soc. Mass. Spectrom*. **1998**, *9*, 225-233.

[2] Alexander, W. M.; Ficarro, S. B.; Adelmant, G.; Marto, J. A., multiplierz v2.0: A Python-based ecosystem for shared access and analysis of native mass spectrometry data. *PROTEOMICS* **2017**, *17*, 1700091.
[3] Ficarro, S.; Alexander, W.; Marto, J., mzStudio: A Dynamic Digital Canvas for User-Driven Interrogation of Mass Spectrometry Data. *Proteomes* **2017**, *5*, 20.

Compound synthesis, characterization, and spectra

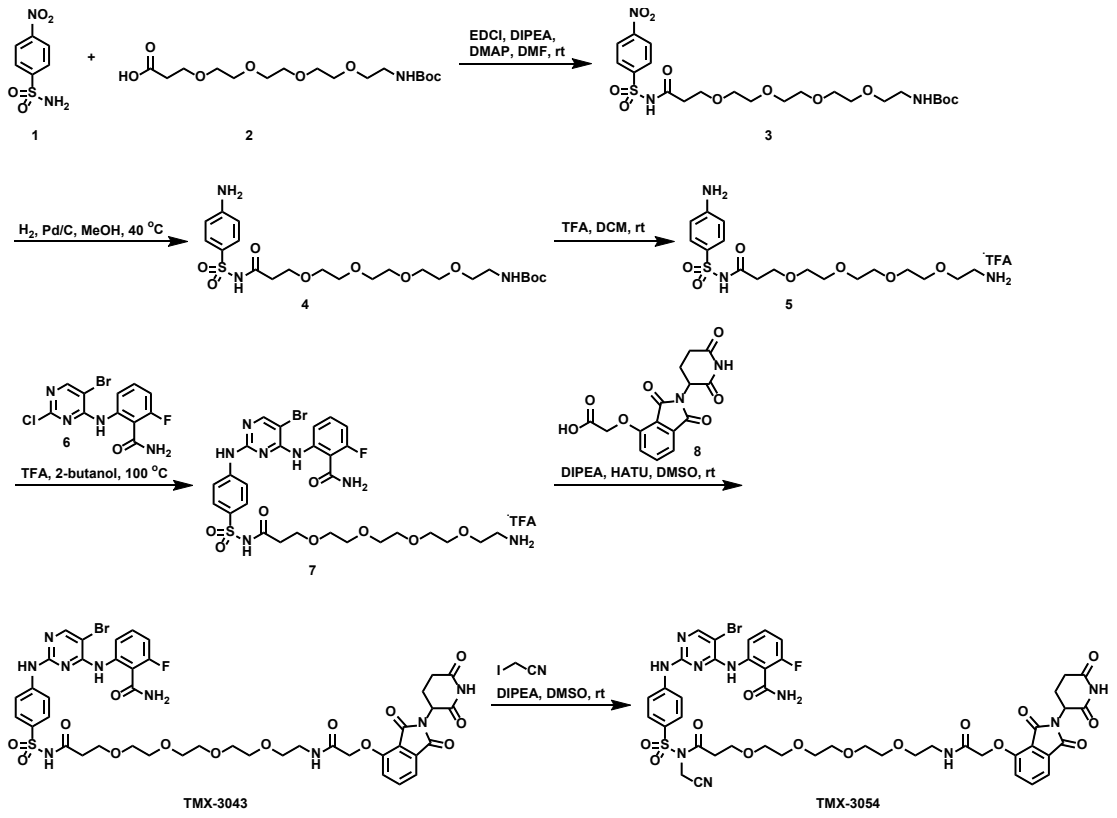
General information

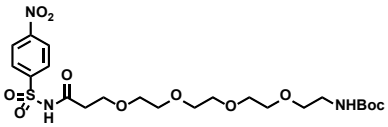
Unless otherwise noted, reagents and solvents were obtained from commercial suppliers and were used without further purification. ^{1}H NMR spectra were recorded on 500 MHz Bruker Avance III spectrometer, and chemical shifts are reported in parts per million (ppm, d) downfield from tetramethylsilane (TMS). Coupling constants (J) are reported in Hz. Spin multiplicities are described as s (singlet), brs (broad singlet), d (doublet), t (triplet), q (quartet), and m (multiplet). Mass spectra were obtained on a Waters Acquity UPLC. Preparative HPLC was performed on a Waters Sunfire C18 column (19 mm X 50 mm, 5 µM) using a gradient of 15-95% methanol in water containing 0.05% trifluoroacetic acid (TFA) over 60 min at a flow rate of 43 mL/min. Flash column chromatography was carried out using prepacked silica cartridges (from 4 g up to 24 g) from Redisep TM and eluted using an Isco Companion system. Purities of assayed compounds were in all cases greater than 95%, as determined by reverse-phase HPLC analysis.

Abbreviations used

EDCI, 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide; DIPEA, N,N-diisopropylethylamine; DMAP, 4-(dimethylamino)pyridine; DMF, N,N-dimethylformamide; MeOH, methanol; TFA, trifluoroacetic acid; DCM, dichloromethane; HATU, hexafluorophosphate azabenzotriazole tetramethyl uronium; DMSO, dimethyl sulfoxide; EtOAc, ethyl acetate; rt, room temperature.

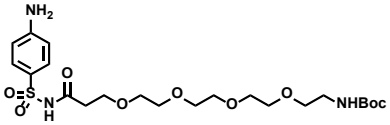
Experimental details for individual compound synthesis





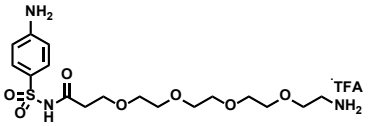
tert-butyl (15-((4-nitrophenyl)sulfonamido)-15-oxo-3,6,9,12-tetraoxapentadecyl)carbamate (3)

To a solution of 4-nitrobenzenesulfonamide **1** (110.0 mg, 0.54 mmol) and *t*-Boc-N-amido-PEG4-acid **2** (198.8 mg, 0.54 mmol) in DMF (2.5 mL) was added DIPEA (0.28 mL, 1.63 mmol), EDCI (126 mg, 0.82 mmol), and DMAP (66 mg, 0.54 mmol). The reaction mixture was stirred at room temperature for 18 hours. The reaction mixture was purified directly via prep HPLC to give **3** (290.0 mg, 97% yield) as a yellow viscous oil. MS (ESI) for $C_{22}H_{36}N_3O_{11}S$ $[M+H]^+$: m/z calcd, 550.21; found, 450.22 (-Boc); MS (ESI) for $C_{22}H_{35}N_3NaO_{11}S$ $[M+Na]^+$: m/z calcd, 572.19; found, 572.16.



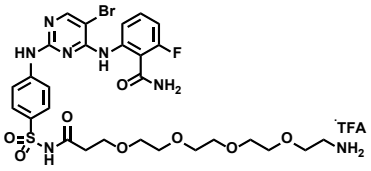
***tert*-butyl (15-((4-aminophenyl)sulfonamido)-15-oxo-3,6,9,12-tetraoxapentadecyl)carbamate (4)**

To a solution of intermediate **3** (290.0 mg, 0.52 mmol) in MeOH (6.0 mL) was added Pd/C (10 wt. % loading, 30.0 mg). The reaction mixture was evacuated and filled with H_2 three times. The mixture was then stirred and hydrogenated (1 bar H_2 pressure) at 40 °C for 2 hours. The reaction mixture was concentrated in vacuo to give **4**, which was used directly in the next step without further purification. ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.62 (s, 1H), 7.52 (d, *J* = 9.0 Hz, 2H), 6.74 (t, *J* = 5.5 Hz, 1H), 6.59 (d, *J* = 9.0 Hz, 2H), 6.12 (s, 2H), 3.53-3.45 (m, 10H), 3.44-3.35 (m, 6H), 3.05 (q, *J* = 6.0 Hz, 2H), 2.39 (t, *J* = 6.0 Hz, 2H), 1.37 (s, 9H). MS (ESI) for $C_{22}H_{38}N_3O_9S$ $[M+H]^+$: m/z calcd, 520.23; found, 520.39.



1-amino-*N*-((4-aminophenyl)sulfonyl)-3,6,9,12-tetraoxapentadecan-15-amide, TFA salt (5)

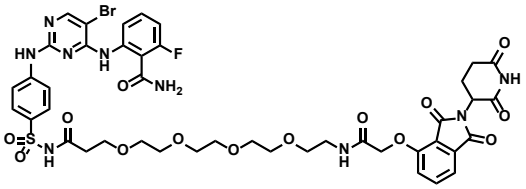
To a solution of intermediate **4** (274.0 mg, 0.52 mmol) in DCM (2.0 mL) was added TFA (0.4 mL). The reaction mixture was stirred at room temperature for 20 minutes. The reaction mixture was concentrated in vacuo to give **5** as a TFA salt, which was used directly in the next step without further purification. MS (ESI) for $C_{17}H_{30}N_3O_7S$ $[M+H]^+$: m/z calcd, 420.18; found, 420.43.



1-amino-*N*-((4-((5-bromo-4-((2-carbamoyl-3-fluorophenyl)amino)pyrimidin-2-yl)amino)phenyl)

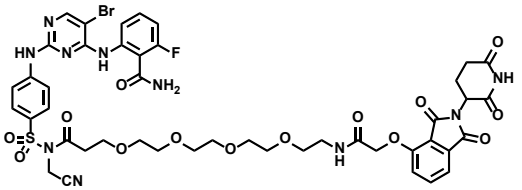
sulfonyl)-3,6,9,12-tetraoxapentadecan-15-amide, TFA salt (7)

A mixture of intermediate **5** (221.0 mg, 0.52 mmol), intermediate **6** (182.0 mg, 0.52 mmol), and TFA (0.1 mL) in 2-butanol (3.0 mL) was heated at 100 °C for 18 hours. The reaction mixture was purified directly via column chromatography (silica gel, eluted with 0% to 10% MeOH in DCM) and prep HPLC sequentially to give **7** (150.0 mg, 33% yield) as a yellow viscous oil. ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.93 (s, 1H), 10.13 (s, 1H), 10.04 (s, 1H), 8.40 (s, 1H), 8.23 (d, *J* = 8.0 Hz, 1H), 8.16 (s, 1H), 8.11 (s, 1H), 7.87 (d, *J* = 9.5 Hz, 2H), 7.81-7.64 (brs, 3H), 7.75 (d, *J* = 8.5 Hz, 2H), 7.53 (td, *J* = 8.5, 6.0 Hz, 1H), 7.11 (t, *J* = 9.0 Hz, 1H), 3.59-3.49 (m, 10H), 3.48-3.43 (m, 6H), 3.00-2.93 (m, 2H), 2.43 (t, *J* = 6.0 Hz, 2H). MS (ESI) for $C_{28}H_{36}BrFN_7O_8S$ $[M+H]^+$: m/z calcd, 728.15, 730.15; found, 728.69, 730.61.



2-((5-bromo-2-((4-(*N*-(1-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)oxy)-2-oxo-6,9,12,15-tetraoxa-3-azaoctadecan-18-oyl)sulfamoyl)phenyl)amino)pyrimidin-4-yl)amino)-6-fluorobenzamide (TMX-3043)

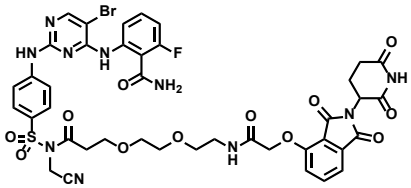
To a solution of intermediate **7** (29.0 mg, 0.034 mmol) and intermediate **8** (11.4 mg, 0.034 mmol) in DMSO (1.5 mL) was added DIPEA (30 µL, 0.17 mmol) and HATU (20 mg, 0.051 mmol). The reaction mixture was stirred at room temperature for 10 minutes. The reaction mixture was purified directly via prep HPLC to give **TMX-3043** (15.0 mg, 41% yield) as a white solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.92 (s, 1H), 11.11 (s, 1H), 10.12 (s, 1H), 10.02 (s, 1H), 8.39 (s, 1H), 8.22 (d, *J* = 8.5 Hz, 1H), 8.15 (s, 1H), 8.11 (s, 1H), 7.94 (t, *J* = 5.5 Hz, 1H), 7.86 (d, *J* = 9.0 Hz, 2H), 7.80 (dd, *J* = 8.0, 7.0 Hz, 1H), 7.74 (d, *J* = 9.0 Hz, 2H), 7.56-7.50 (m, 1H), 7.49 (d, *J* = 7.0 Hz, 1H), 7.38 (d, *J* = 8.5 Hz, 1H), 7.10 (t, *J* = 9.0 Hz, 1H), 5.11 (dd, *J* = 12.5, 5.5 Hz, 1H), 4.78 (s, 2H), 3.53-3.35 (m, 16H), 3.33-3.28 (m, 2H), 2.93-2.84 (m, 1H), 2.63-2.51 (m, 2H), 2.43 (t, *J* = 6.0 Hz, 2H), 2.07-2.00 (m, 1H). MS (ESI) for $C_{43}H_{46}BrFN_9O_{14}S$ $[M+H]^+$: m/z calcd, 1042.21, 1044.20; found, 1042.77, 1044.80.



2-((5-bromo-2-((4-(*N*-(cyanomethyl)-*N*-(1-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)oxy)-2-oxo-6,9,12,15-tetraoxa-3-azaoctadecan-18-oyl)sulfamoyl)phenyl)amino)pyrimidin-4-yl)amino)-6-fluorobenzamide (TMX-3054)

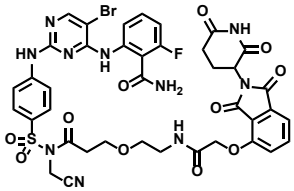
To a solution of **TMX-3043** (15.0 mg, 0.014 mmol) in DMSO (1.5 mL) was added DIPEA (50 µL, 0.29 mmol) and 2-iodoacetonitrile (24.0 mg, 0.14 mmol). The reaction mixture was stirred at room temperature for 40 minutes. The reaction mixture was purified directly via prep HPLC to

give **TMX-3054** (3.8 mg, 24% yield) as a white solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.11 (s, 1H), 10.18 (s, 1H), 10.15 (s, 1H), 8.42 (s, 1H), 8.22 (d, *J* = 8.5 Hz, 1H), 8.16 (s, 1H), 8.11 (s, 1H), 7.99 (t, *J* = 5.5 Hz, 1H), 7.95 (d, *J* = 9.5 Hz, 2H), 7.86 (d, *J* = 9.0 Hz, 2H), 7.80 (dd, *J* = 8.5, 7.5 Hz, 1H), 7.58-7.51 (m, 1H), 7.48 (d, *J* = 7.5 Hz, 1H), 7.38 (d, *J* = 8.5 Hz, 1H), 7.09 (t, *J* = 8.5 Hz, 1H), 5.11 (dd, *J* = 12.5, 5.0 Hz, 1H), 4.91 (s, 2H), 4.77 (s,2 H), 3.49-3.39 (m, 16H), 3.33-3.27 (m, 2H), 2.93 (t, *J* = 6.0 Hz, 2H), 2.90-2.84 (m, 1H), 2.63-2.51 (m, 2H), 2.07-1.99 (m, 1H). MS (ESI) for $C_{45}H_{47}BrFN_{10}O_{14}S$ $[M+H]^+$: m/z calcd, 1081.22, 1083.21; found, 1081.93, 1083.79.



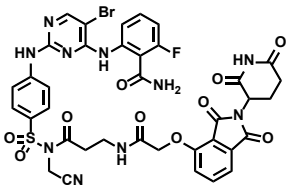
2-((5-bromo-2-((4-(N-(cyanomethyl)-N-(3-(2-(2-(2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)oxy)acetamido)ethoxy)ethoxy)propanoyl)sulfamoyl)phenyl)amino)pyrimidin-4-yl)amino)-6-fluorobenzamide (TMX-3166)

Following the same procedure as compound **TMX-3054**, compound **TMX-3166** (3.5 mg, 25% yield) was prepared as a white solid. (500 MHz, DMSO-d_6) δ 11.10 (s, 1H), 10.17 (s, 1H), 10.15 (s, 1H), 8.41 (s, 1H), 8.22 (d, *J* = 8.5 Hz, 1H), 8.16 (s, 1H), 8.10 (s, 1H), 7.98-7.92 (m, 3H), 7.86 (d, *J* = 9.0 Hz, 2H), 7.80 (dd, *J* = 8.5, 7.5 Hz, 1H), 7.57-7.51 (m, 1H), 7.47 (d, *J* = 7.5 Hz, 1H), 7.36 (d, *J* = 8.5 Hz, 1H), 7.09 (t, *J* = 9.0 Hz, 1H), 5.10 (dd, *J* = 13.0, 6.0 Hz, 1H), 4.90 (s, 2H), 4.76 (s,2 H), 3.44 (s, 4H), 3.41 (t, *J* = 5.5 Hz, 2H), 3.27 (dd, *J* = 11.5, 5.5 Hz, 2H), 2.93 (t, *J* = 6.0 Hz, 2H), 2.90-2.84 (m, 1H), 2.65-2.51 (m, 2H), 2.06-1.99 (m, 1H). MS (ESI) for $C_{41}H_{39}BrFN_{10}O_{12}S$ $[M+H]^+$: m/z calcd, 993.16, 995.16; found, 993.21, 995.02.



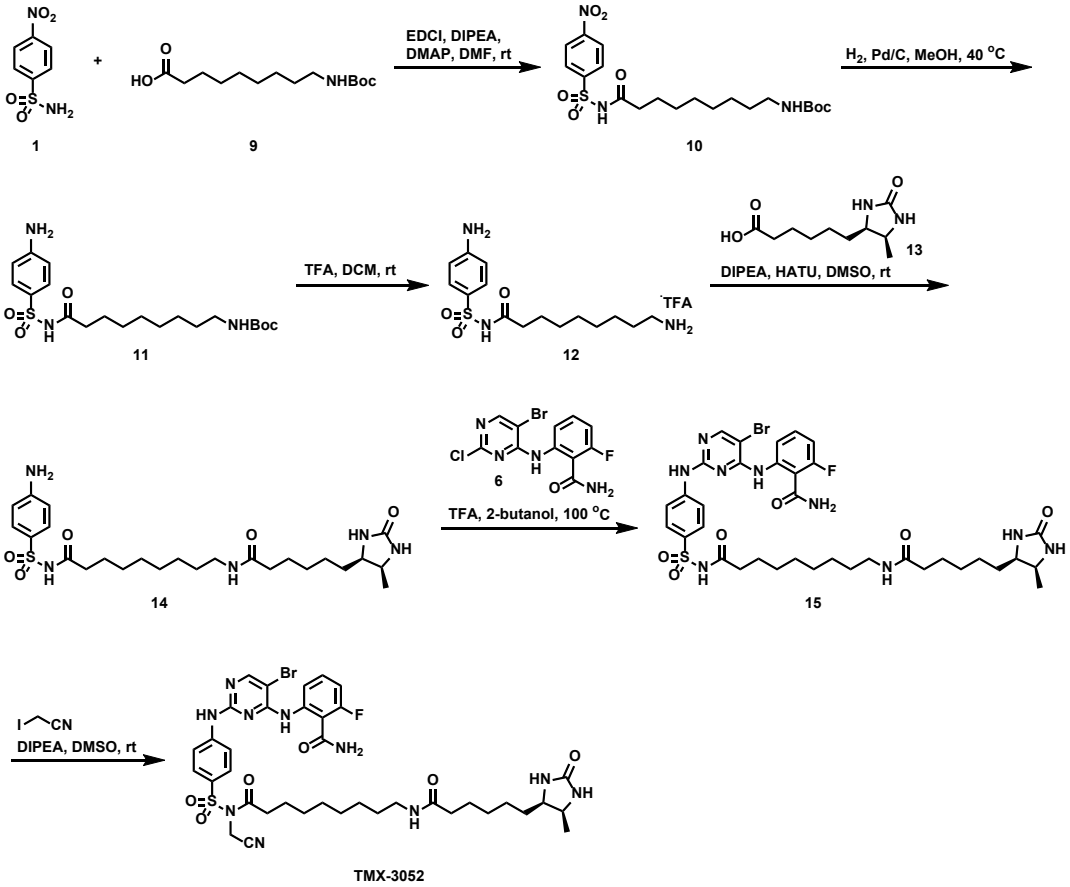
2-((5-bromo-2-((4-(N-(cyanomethyl)-N-(3-(2-(2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)oxy)acetamido)ethoxy)propanoyl)sulfamoyl)phenyl)amino)pyrimidin-4-yl)amino)-6-fluorobenzamide (TMX-3164)

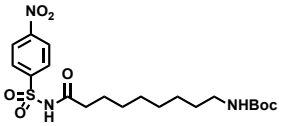
Following the same procedure as compound **TMX-3054**, compound **TMX-3164** (5.5 mg, 38% yield) was prepared as a white solid. (500 MHz, DMSO-d_6) δ 11.10 (s, 1H), 10.17 (s, 1H), 10.14 (s, 1H), 8.40 (s, 1H), 8.21 (d, *J* = 8.0 Hz, 1H), 8.16 (s, 1H), 8.10 (s, 1H), 7.97-7.91 (m, 3H), 7.86 (d, *J* = 9.5 Hz, 2H), 7.77 (dd, *J* = 8.0, 7.5 Hz, 1H), 7.57-7.51 (m, 1H), 7.46 (d, *J* = 6.5 Hz, 1H), 7.34 (d, *J* = 8.5 Hz, 1H), 7.08 (t, *J* = 9.5 Hz, 1H), 5.10 (dd, *J* = 13.9, 5.5 Hz, 1H), 4.90 (s, 2H), 4.75 (s,2 H), 3.62 (t, *J* = 6.0 Hz, 2H), 3.38 (t, *J* = 5.0 Hz, 2H), 3.25 (dd, *J* = 11.0, 5.5 Hz, 2H), 2.96 (t, *J* = 6.0 Hz, 2H), 2.92-2.82 (m, 1H), 2.65-2.51 (m, 2H), 2.06-1.99 (m, 1H). MS (ESI) for $C_{39}H_{35}BrFN_{10}O_{11}S$ $[M+H]^+$: m/z calcd, 949.14, 951.14; found, 949.15, 951.01.



2-((5-bromo-2-((4-(N-(cyanomethyl)-N-(3-(2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)oxy)acetamido)propanoyl)sulfamoyl)phenyl)amino)pyrimidin-4-yl)amino)-6-fluorobenzamide (TMX-3185)

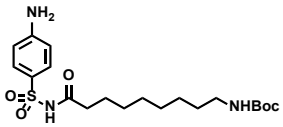
Following the same procedure as compound **TMX-3054**, compound **TMX-3185** (3.0 mg, 35% yield) was prepared as a white solid. (500 MHz, DMSO-d_6) δ 11.11 (s, 1H), 10.17 (s, 1H), 10.12 (s, 1H), 8.40 (s, 1H), 8.20 (d, *J* = 8.5 Hz, 1H), 8.19 (s, 1H), 8.11 (s, 1H), 8.00 (t, *J* = 5.5 Hz, 1H), 7.93 (d, *J* = 9.0 Hz, 2H), 7.85 (d, *J* = 9.0 Hz, 2H), 7.73 (dd, *J* = 8.0, 6.0 Hz, 1H), 7.59-7.52 (m, 1H), 7.44 (d, *J* = 7.5 Hz, 1H), 7.30 (d, *J* = 8.5 Hz, 1H), 7.08 (t, *J* = 9.0 Hz, 1H), 5.09 (dd, *J* = 13.0, 5.0 Hz, 1H), 4.87 (s, 2H), 4.70 (s, 2H), 3.33-3.25 (m, 2H), 2.95 (t, *J* = 6.0 Hz, 2H), 2.93-2.83 (m, 1H), 2.63-2.51 (m, 2H), 2.06-1.98 (m, 1H). MS (ESI) for $C_{37}H_{31}BrFN_{10}O_{10}S$ $[M+H]^+$: m/z calcd, 905.11, 907.11; found, 905.08, 907.00.





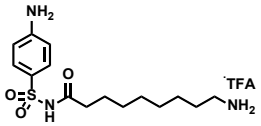
***tert*-butyl (9-((4-nitrophenyl)sulfonamido)-9-oxononyl)carbamate (10)**

By using 4-nitrobenzenesulfonamide **1** and 9-{[(*tert*-butoxy)carbonyl]amino}nonanoic acid **9**, following the same procedure as compound **3**, compound **10** (100.0 mg, 88% yield) was prepared as a yellow viscous oil. MS (ESI) for $C_{20}H_{32}N_3O_7S$ $[M+H]^+$: m/z calcd, 458.20; found, 358.26 (-Boc).



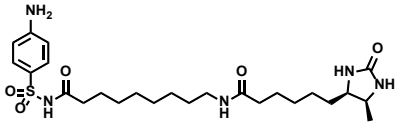
***tert*-butyl (9-((4-aminophenyl)sulfonamido)-9-oxononyl)carbamate (11)**

By using intermediate **10**, following the same procedure as compound **4**, crude **11** was prepared and used directly in the next step without further purification. MS (ESI) for $C_{20}H_{34}N_3O_5S$ $[M+H]^+$: m/z calcd, 428.22; found, 328.31 (-Boc).



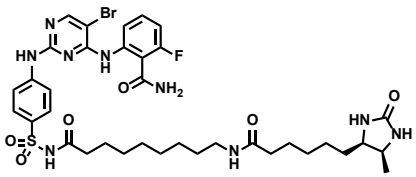
9-amino-*N*-((4-aminophenyl)sulfonyl)nonanamide, TFA salt (12)

By using intermediate **11**, following the same procedure as compound **5**, crude **12** was prepared and used directly in the next step without further purification. ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.56 (s, 1H), 7.60 (brs, 3H), 7.51 (d, *J* = 9.0 Hz, 2H), 6.59 (d, *J* = 9.0 Hz, 2H), 6.12 (s, 2H), 2.79-2.71 (m, 2H), 2.12 (t, *J* = 7.5 Hz, 2H), 1.53-1.45 (m, 2H), 1.42-1.35 (m, 2H), 1.28-1.11 (m, 8H). MS (ESI) for $C_{15}H_{26}N_3O_3S$ $[M+H]^+$: m/z calcd, 328.17; found, 328.31.



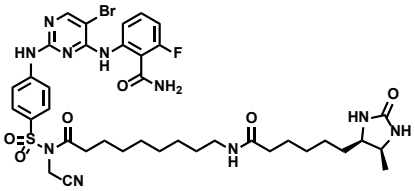
***N*-((4-aminophenyl)sulfonyl)-9-(6-((4*R*,5*S*)-5-methyl-2-oxoimidazolidin-4-yl)hexanamido)nonanamide (14)**

By using intermediate **12** and *d*-desthiobiotin **13**, following the same procedure as compound **TMX-3043**, compound **14** (150.0 mg, 93% yield) was prepared as a yellow viscous oil. ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.55 (s, 1H), 7.69 (t, *J* = 5.5 Hz, 1H), 7.51 (d, *J* = 9.0 Hz, 2H), 6.59 (d, *J* = 8.5 Hz, 2H), 6.29 (brs, 2H), 6.11 (brs, 2H), 3.63-3.56 (m, 1H), 3.49-3.43 (m, 1H), 2.99 (q, *J* = 6.5 Hz, 2H), 2.12 (t, *J* = 7.5 Hz, 2H), 2.02 (t, *J* = 7.5 Hz, 2H), 1.50-1.43 (m, 2H), 1.41-1.28 (m, 7H), 1.25-1.08 (m, 11H), 0.95 (d, *J* = 6.5 Hz, 3H). MS (ESI) for $C_{25}H_{42}N_5O_5S$ $[M+H]^+$: m/z calcd, 524.29; found, 524.58.



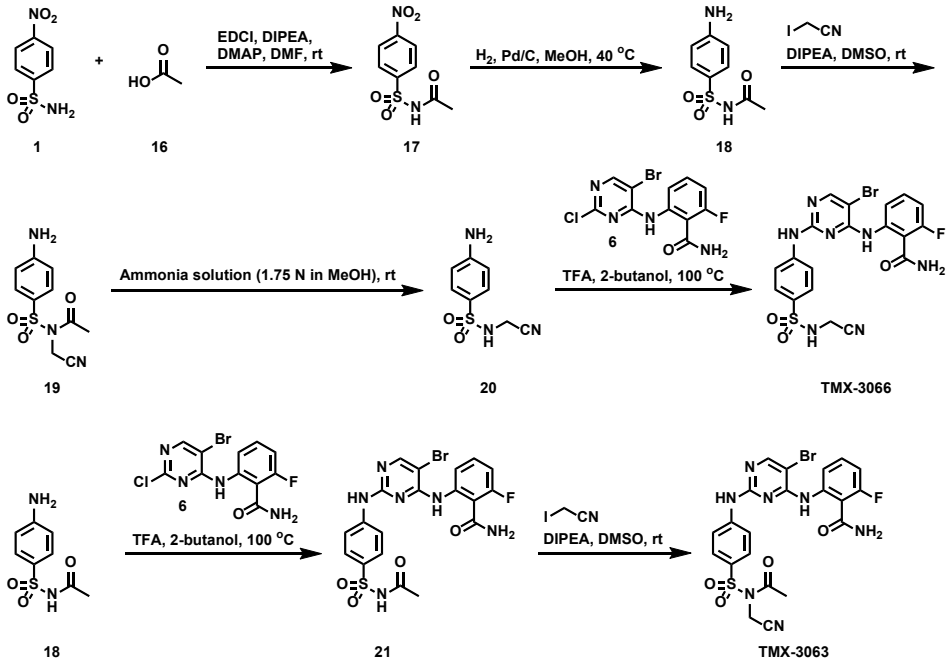
2-((5-bromo-2-((4-(*N*-(9-(6-((4*R*,5*S*)-5-methyl-2-oxoimidazolidin-4-yl)hexanamido)nonanoyl)sulfamoyl)phenyl)amino)pyrimidin-4-yl)amino)-6-fluorobenzamide (15)

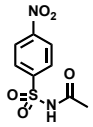
By using intermediate **14** and intermediate **6**, following the same procedure as compound **7**, compound **15** (10.0 mg, 18% yield) was prepared as a white solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.84 (s, 1H), 10.12 (s, 1H), 10.03 (s, 1H), 8.40 (s, 1H), 8.22 (d, *J* = 8.5 Hz, 1H), 8.16 (s, 1H), 8.11 (s, 1H), 7.86 (d, *J* = 8.5 Hz, 2H), 7.74 (d, *J* = 9.0 Hz, 2H), 7.66 (t, *J* = 5.5 Hz, 1H), 7.56-7.50 (m, 1H), 7.10 (t, *J* = 9.0 Hz, 1H), 6.28 (s, 1H), 6.10 (s, 1H), 3.63-3.56 (m, 1H), 3.49-3.43 (m, 1H), 2.96 (q, *J* = 6.5 Hz, 2H), 2.16 (t, *J* = 7.5 Hz, 2H), 2.01 (t, *J* = 7.5 Hz, 2H), 1.49-1.42 (m, 2H), 1.40-1.27 (m, 7H), 1.24-1.06 (m, 11H), 0.94 (d, *J* = 6.5 Hz, 3H). MS (ESI) for $C_{36}H_{48}BrFN_9O_6S$ $[M+H]^+$: m/z calcd, 832.26, 834.26; found, 832.72, 834.70.



2-((5-bromo-2-((4-(*N*-(cyanomethyl)-*N*-(9-(6-((4*R*,5*S*)-5-methyl-2-oxoimidazolidin-4-yl)hexanamido)nonanoyl)sulfamoyl)phenyl)amino)pyrimidin-4-yl)amino)-6-fluorobenzamide (TMX-3052)

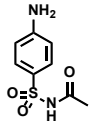
By using intermediate **15** and 2-iodoacetonitrile, following the same procedure as compound **TMX-3054**, compound **TMX-3052** (3.5 mg, 33% yield) was prepared as a white solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.19 (s, 1H), 10.17 (s, 1H), 8.43 (s, 1H), 8.22 (d, *J* = 8.5 Hz, 1H), 8.16 (s, 1H), 8.11 (s, 1H), 7.95 (d, *J* = 9.0 Hz, 2H), 7.86 (d, *J* = 9.0 Hz, 2H), 7.66 (t, *J* = 5.5 Hz, 1H), 7.57-7.51 (m, 1H), 7.08 (t, *J* = 9.0 Hz, 1H), 6.40-6.00 (m, 2H), 4.92 (s, 2H), 3.62-3.56 (m, 1H), 3.49-3.43 (m, 1H), 2.96 (q, *J* = 5.2 Hz, 2H), 2.63 (t, *J* = 7.5 Hz, 2H), 2.01 (t, *J* = 7.5 Hz, 2H), 1.49-1.39 (m, 4H), 1.33-1.11 (m, 16H), 0.94 (d, *J* = 6.0 Hz, 3H). MS (ESI) for $C_{38}H_{49}BrFN_{10}O_6S$ $[M+H]^+$: m/z calcd, 871.27, 873.27; found, 871.71, 873.68.





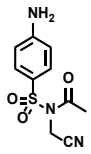
***N*-((4-nitrophenyl)sulfonyl)acetamide (17)**

By using 4-nitrobenzenesulfonamide **1** and acetic acid **16**, following the same procedure as compound **3**, compound **17** (200.0 mg, 94% yield) was prepared as a colorless viscous oil. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.28 (d, *J* = 9.0 Hz, 2H), 8.01 (d, *J* = 9.0 Hz, 2H), 1.74 (s, 3H) (*The exchangeable proton of -$SO_2NHCOCH_3$ is missing in the spectrum*). MS (ESI) for $C_8H_8N_2NaO_5S$ $[M+Na]^+$: m/z calcd, 267.01; found, 267.02.



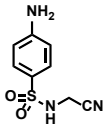
***N*-((4-aminophenyl)sulfonyl)acetamide (18)**

By using intermediate **17**, following the same procedure as compound **4**, crude **11** was prepared as a colorless viscous oil, which was used directly in the next step without further purification. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.52 (d, *J* = 9.0 Hz, 2H), 6.59 (d, *J* = 8.5 Hz, 2H), 6.12 (s, 2H), 1.86 (s, 3H) (*The exchangeable proton of -$SO_2NHCOCH_3$ is missing in the spectrum*). MS (ESI) for $C_8H_{11}N_2O_3S$ $[M+H]^+$: m/z calcd, 215.05; found, 215.17.



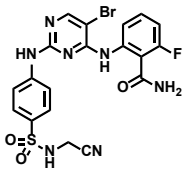
***N*-((4-aminophenyl)sulfonyl)-N-(cyanomethyl)acetamide (19)**

By using intermediate **18** and 2-iodoacetonitrile, following the same procedure as compound **TMX-3054**, compound **19** (45.0 mg, 95% yield) was prepared as a yellow viscous oil. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.62 (d, J = 8.5 Hz, 2H), 6.66 (d, J = 9.0 Hz, 2H), 6.54-5.90 (m, 2H), 4.77 (s, 2H), 2.32 (s, 3H). MS (ESI) for $C_{10}H_{11}N_3NaO_3S$ $[M+Na]^+$: m/z calcd, 276.04; found, 276.02.



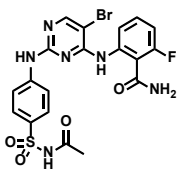
4-amino-*N*-(cyanomethyl)benzenesulfonamide (20)

Intermediate **19** (30.0 mg, 0.12 mmol) was dissolved in ammonia solution (1.75 M in MeOH, 2.0 mL). The reaction mixture was stirred at room temperature for 20 minutes. The reaction mixture was concentrated in vacuo to give **20**, which was used directly in the next step without further purification. MS (ESI) for $C_8H_9N_3NaO_2S$ $[M+Na]^+$: m/z calcd, 234.03; found, 234.03.



2-((5-bromo-2-((4-(*N*-(cyanomethyl)sulfamoyl)phenyl)amino)pyrimidin-4-yl)amino)-6-fluorobenzamide (TMX-3066)

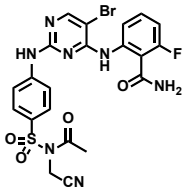
By using intermediate **20** and intermediate **6**, following the same procedure as compound **7**, compound **TMX-3066** (3.0 mg, 5% yield) was prepared as a white solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.10 (s, 1H), 10.00 (s, 1H), 8.40 (s, 1H), 8.35 (t, J = 6.0 Hz, 1H), 8.23 (d, J = 8.5 Hz, 1H), 8.16 (s, 1H), 8.12 (s, 1H), 7.88 (d, J = 9.0 Hz, 2H), 7.67 (d, J = 8.5 Hz, 2H), 7.58-7.50 (m, 1H), 7.13-7.06 (m, 1H), 4.05 (d, J = 6.0 Hz, 2H). MS (ESI) for $C_{19}H_{16}BrFN_7O_3S$ $[M+H]^+$: m/z calcd, 520.02, 522.02; found, 520.19, 522.16.



2-((2-((4-(*N*-acetylsulfamoyl)phenyl)amino)-5-bromopyrimidin-4-yl)amino)-6-fluorobenzamide (21)

By using intermediate **18** and intermediate **6**, following the same procedure as compound **7**, compound **21** (6.3 mg, 5% yield) was prepared as a white solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.90 (s, 1H), 10.10 (s, 1H), 10.02 (s, 1H), 8.40 (s, 1H), 8.20 (d, J = 8.0 Hz, 1H), 8.15 (s, 1H), 8.11 (s, 1H), 7.86 (d, J = 9.0 Hz, 2H), 7.74 (d, J = 8.5 Hz, 2H), 7.56-7.49 (m, 1H), 7.13-7.07 (m, 1H), 1.90 (s,

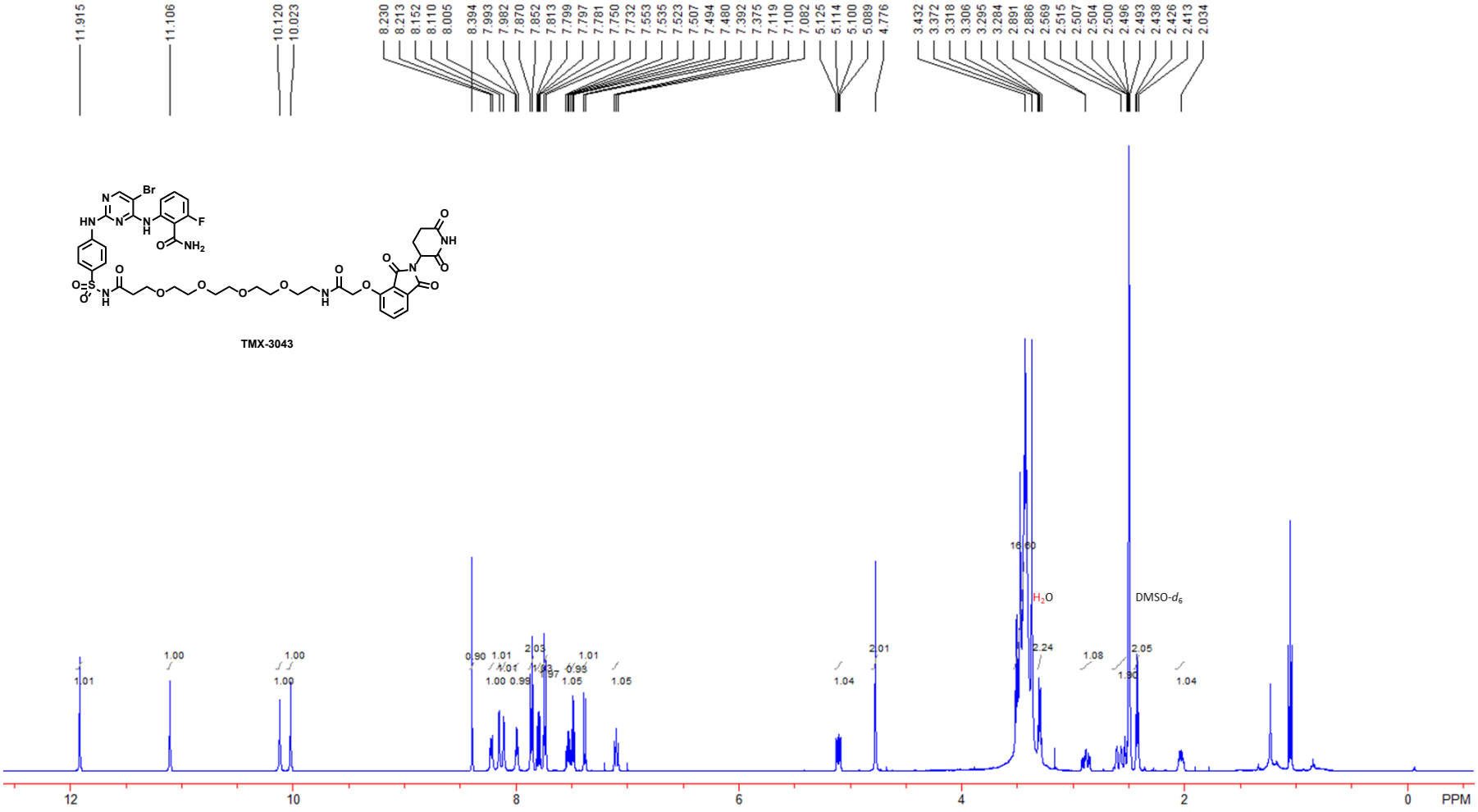
3H). MS (ESI) for $C_{19}H_{17}BrFN_6O_4S$ $[M+H]^+$: m/z calcd, 523.02, 525.02; found, 523.39, 525.31.

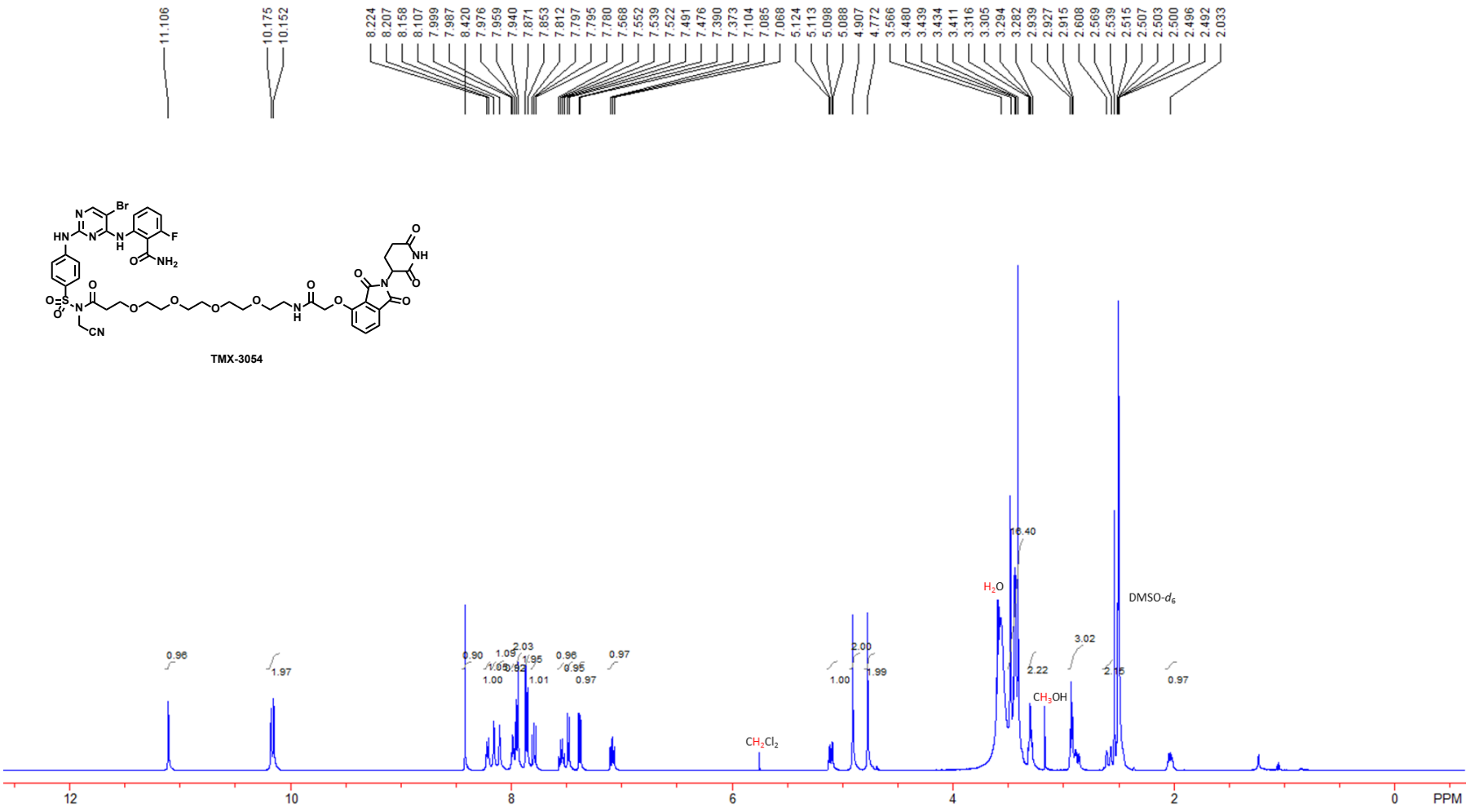


2-((2-((4-(*N*-acetyl-*N*-(cyanomethyl)sulfamoyl)phenyl)amino)-5-bromopyrimidin-4-yl)amino)-6-fluorobenzamide (TMX-3063)

By using intermediate **21** and 2-iodoacetonitrile, following the same procedure as compound **TMX-3054**, compound **TMX-3063** (2.2 mg, 40% yield) was prepared as a white solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.16 (s, 1H), 10.16 (s, 1H), 8.43 (s, 1H), 8.21 (d, *J* = 8.5 Hz, 1H), 8.16 (s, 1H), 8.11 (s, 1H), 7.96 (d, *J* = 9.0 Hz, 2H), 7.87 (d, *J* = 8.5 Hz, 2H), 7.58-7.52 (m, 1H), 7.09 (t, *J* = 9.5 Hz, 2H), 4.88 (s, 2H), 2.34 (s, 3H). MS (ESI) for $C_{21}H_{18}BrFN_7O_4S$ $[M+H]^+$: m/z calcd, 562.03, 564.03; found, 562.38, 564.35.

Spectra of key compounds

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7.795
7.780
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7.552
7.539
7.522
7.491
7.476
7.390
7.373
7.104
7.085
7.068
5.124
5.113
5.098
5.088
4.907
4.772
3.566
3.480
3.439
3.434
3.411
3.316
3.305
3.294
3.282
2.939
2.927
2.915
2.608
2.569
2.539
2.515
2.507
2.503
2.500
2.496
2.492
2.033
TMX-3054
0.96
1.97
0.90
1.09
2.03
1.95
0.96
0.97
1.00
1.01
0.95
0.97
CH2Cl2
1.00
2.00
1.99
H2O
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2.22
3.02
CH3OH
DMSO-d6
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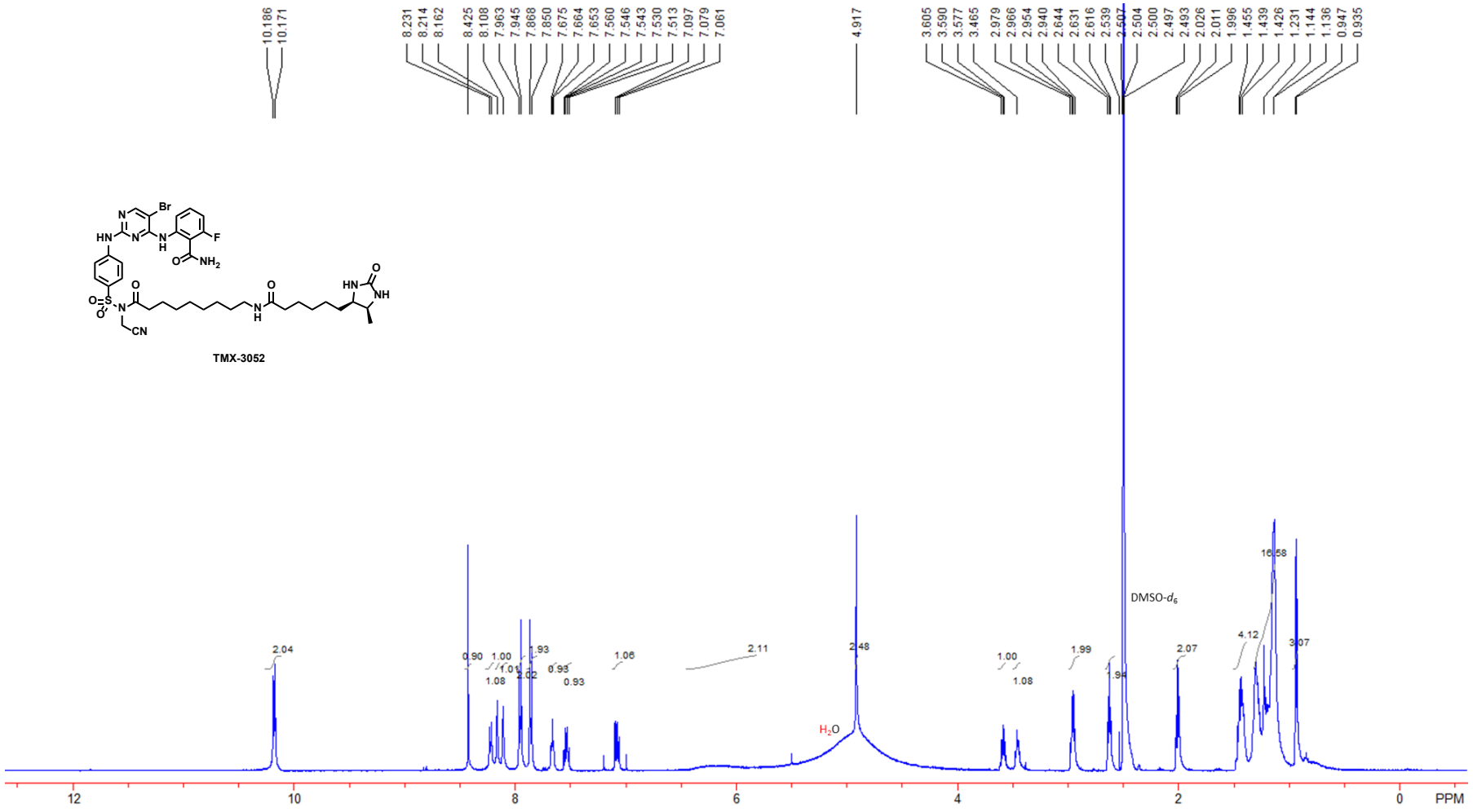
0.97
12
10
8
6
4
2
0
PPM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7.653
7.560
7.546
7.543
7.530
7.513
7.097
7.079
7.061
4.917
3.605
3.590
3.577
3.465
2.979
2.966
2.954
2.940
2.644
2.631
2.616
2.539
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2.504
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2.493
2.026
2.011
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1.455
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1.426
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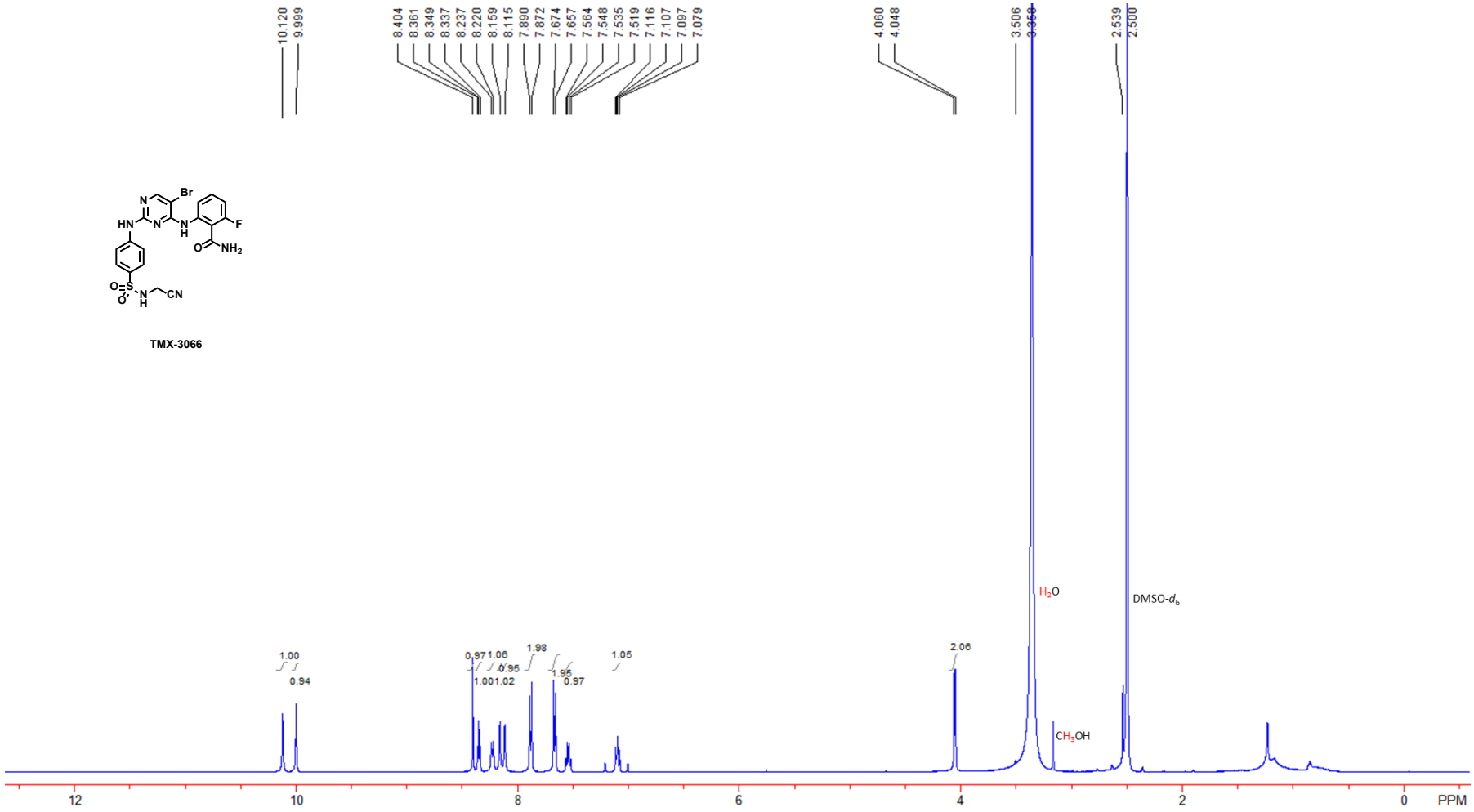
1.144
1.136
0.947
0.935
TMX-3052
DMSO-d_6
H_2O
PPM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7.548
7.535
7.519
7.116
7.107
7.097
7.079
4.060
4.048
3.506
3.366
2.539
2.500
TMX-3066
1.00
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0.97
1.06
0.95
1.00
1.02
1.98
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0.97
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2.06
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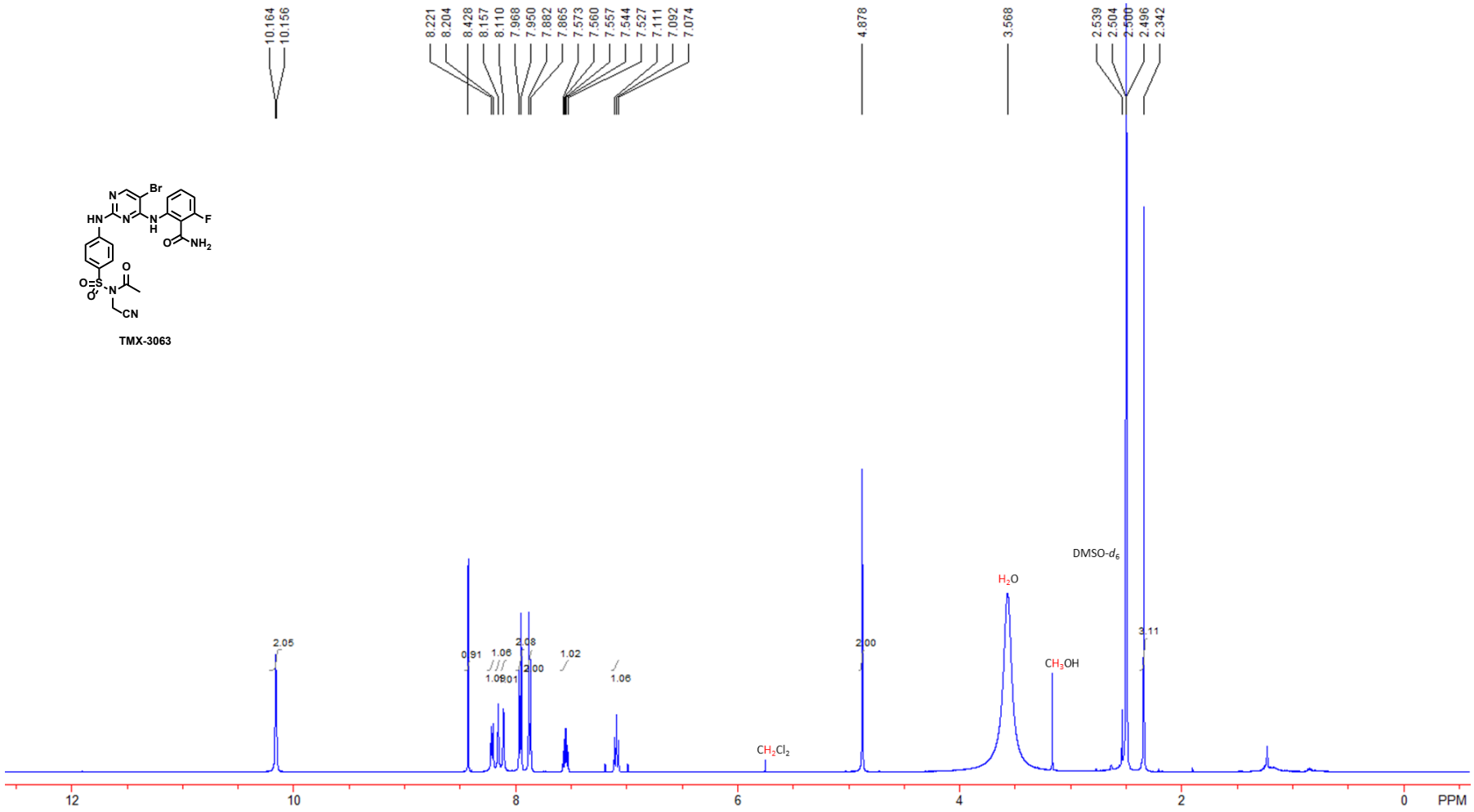
CH3OH
DMSO-d6
12
10
8
6
4
2
0
PPM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7.111
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7.074
4.878
3.568
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2.496
2.342
TMX-3063
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1.02
1.06
2.00
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CH3OH
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