Supporting information for:

Heterobifunctional molecules induce dephosphorylation of kinases – a proof of concept study

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(a) (b)

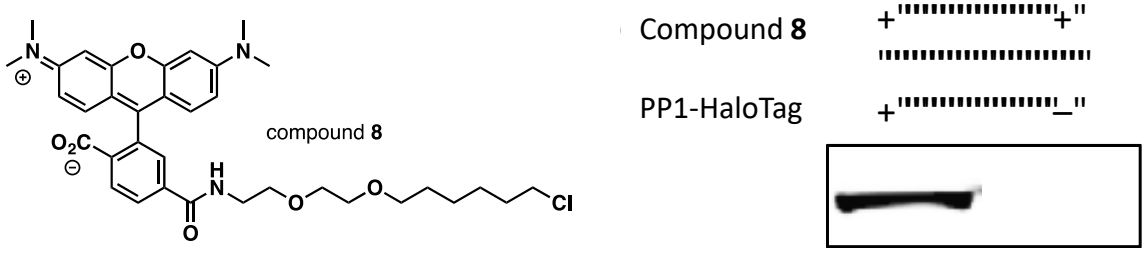


Figure S1. (a) Chloroalkyl-TAMRA compound **8**; (b) In-gel fluorescence of FLAG-PP1-HaloTag with and without treatment with 1 μM **8**. (LNCaP/ner cells, 1 hour treatment).

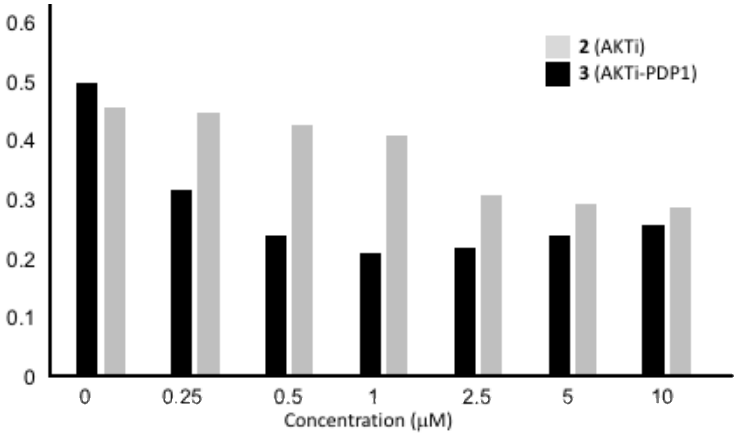


Figure S2. Allosteric AKT inhibitor-PDP1 decreased pAKT^{T308} levels in the presence of PP1. pAKT^{T308} levels after 1 hr incubation of PP1, pAKT, and **2** or **3**. In this experiment, I2 was not included.

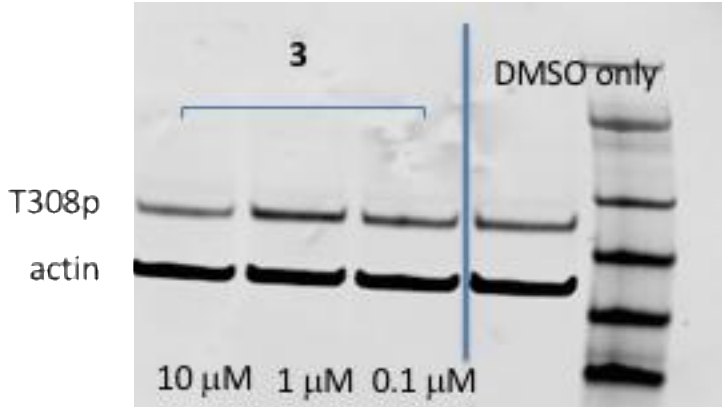


Figure S3. Compound **3** failed to exhibit strong dephosphorylating activity in the cells. Immunoblot analysis of pAkt^{t308} levels in LNCaP/ner cells treated with compound **3** for 2 hours.

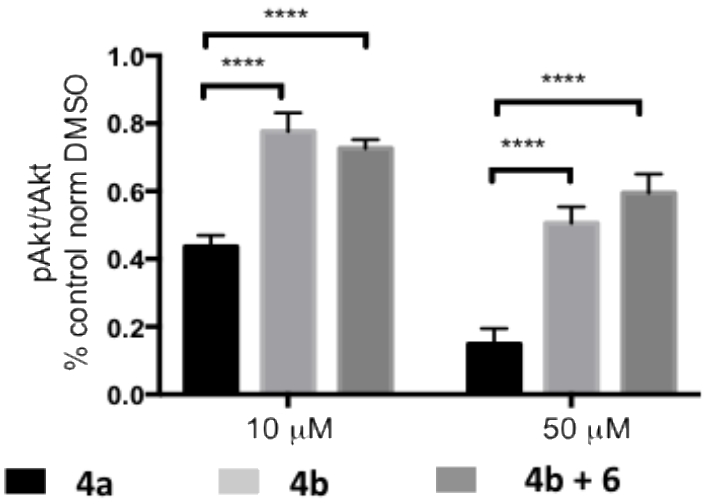


Figure S4. Quantification of pAKT^{S473}/AKT relative to DMSO control in LNCaP cells for 8 hr at indicated concentrations. NS: P > 0.05, *: P ≤ 0.05, **: P ≤ 0.01, ***: P ≤ 0.001, ***: P ≤ 0.0001.

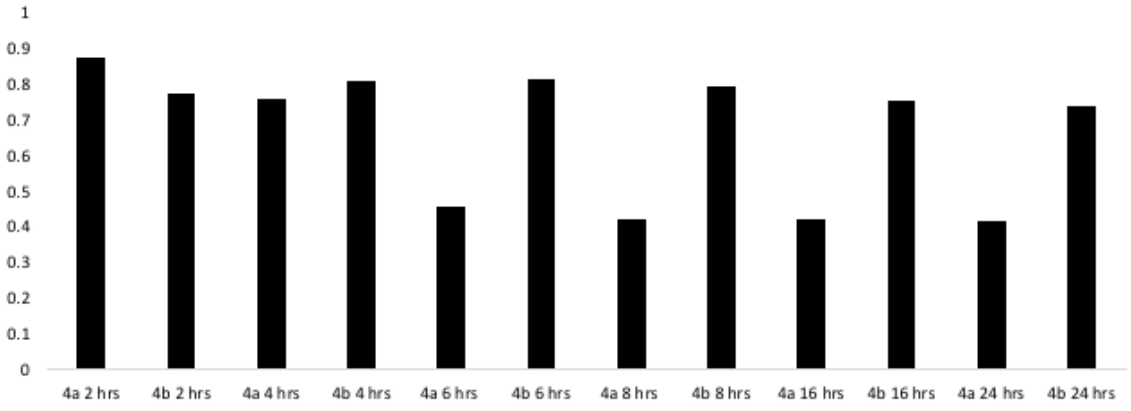


Figure S5. Timecourse immunoblot analysis and quantification of pAkt^{t308} levels after normalization over total Akt in LNCaP/ner cells treated with **4a** (10 μM) or **4b** (10 μM).

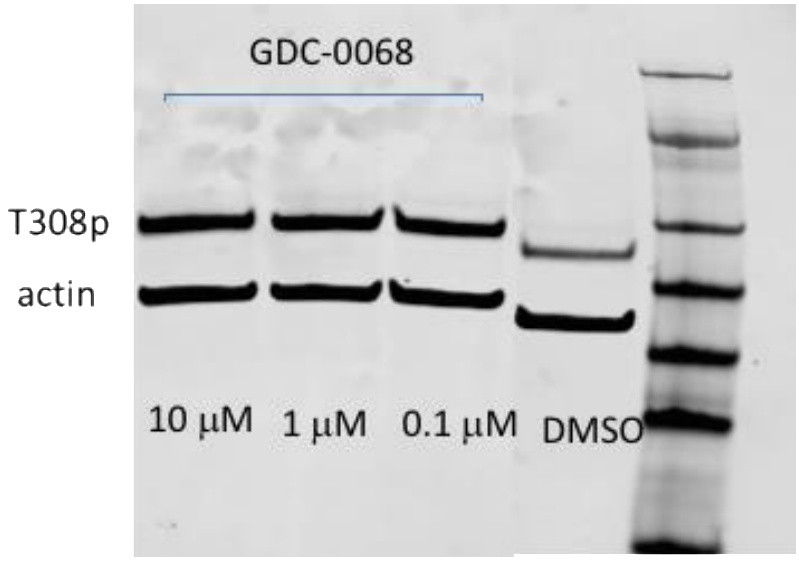


Figure S6. ATP competitive AKT inhibitor protects pAKT from phosphatase. Immunoblot analysis of $pAkt^{t308}$ levels in LNCaP/ner cells treated with compound active-site AKT inhibitor for 2 hours.

Biological methods

Reagents. Phospho-Akt (Ser472) (D9E) rabbit mAb, phospho-Akt (Thr308) (C31E5E) rabbit mAb, Akt (pan) (40D4) mouse mAb, phospho-EGF receptor (Tyr1068) (D7A5) rabbit mAb, EGF receptor rabbit antibody, PP1a rabbit antibody, and β-actin (13E5) rabbit mAb were purchased from Cell Signaling Technology. Anti-FLAG (F1804) antibody was purchased from Sigma.

Cell Culture. All cell lines were obtained from the Genentech cell line repository and cultured based upon ATCC specifications. LNCaP/ner cells were maintained in RPMI 1640 medium supplemented with FBS (Sigma F2442), and 1X GlutaMax (Gibco 35050-061). MCF7-neo/HER2, PC3, HCC827 cells were further supplemented with 1X Pen Strep (Gibco 15140-122). For drug treatment experiments, cells were serum-starved overnight in the presence of media supplemented with GlutaMAX.

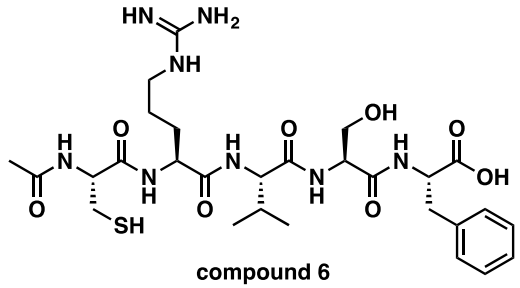
Constructs. N-HaloTag, C-FLAG-fused Human PPP1CA was cloned into a pRK5 expression vector. Cells were transfected using Lipofectamine LTX, PLUS reagents and Opti-MEM (Gibco 31985-070) using standard protocols 24 hours prior to drug treatment. Pooled siRNAs targeting human PPP1CA were purchased from GE Dharmacon. Cells were transfected using Lipofectamine RNAiMAX reagent and Opti-MEM using reverse transfection protocols 48 hours prior to drug treatment. The amount of DNA, siRNA used was optimized in each condition.

Immunoblot analysis. After overnight serum starvation, LNCaP/ner, MCF7-neo/HER2, PC3 cells were incubated in serum-free media in the presence of 10, or 50 μM of compound and 0.1% DMSO for 8 hours 37C in a CO_2 incubator. Cells were washed with PBS once and lysed in M-PER Mammalian Protein Extraction Reagent (Thermo) supplemented with Halt Protease and Phosphatase inhibitor Cocktail (Thermo) using manufactures recommended protocol.

HCC827 cells were 10 mM Tris pH7.5, 50 mM NaCl, 1 mM EDTA, 0.5% NP40 supplemented with COMPLETE EDTA-free protease inhibitor (Roche), and HALT phosphatase inhibitors (Thermo). Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and detected using a Li-Cor scanner.

Chemistry methods

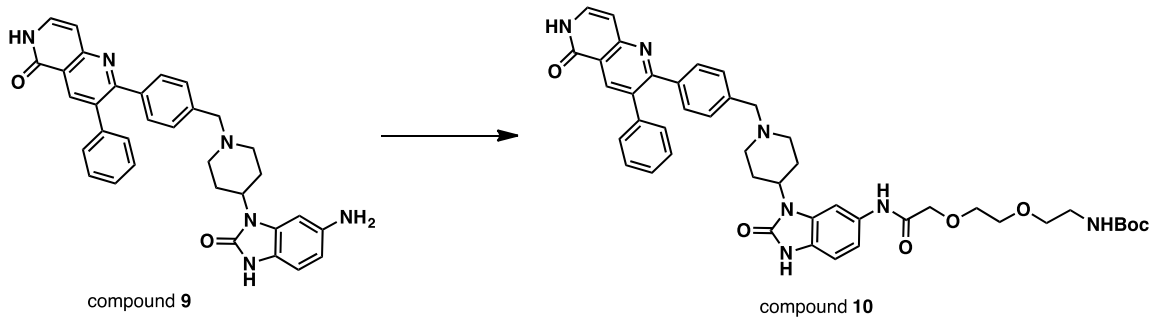
General Synthetic procedures. All solvents and reagents were used as obtained. ^{1}H NMR spectra were recorded with Bruker spectrometers and referenced to tetramethyl silane. Non peptidic molecules were analyzed by HPLC (Waters Acquity UPLC column) with UV detection at 254 and 210 nm, and purified by HPLC (Interchim, Phenomenex Luna-C18, Phenomenex Gemini-NX) or Teledyne ISCO CombiFlash (RediSep Rf silica gel column). Peptides and Peptide-small molecule conjugates were analyzed by HPLC (Waters, Xevo Qtof, UPLC column) with UV detection at 220 and 280 nm, and purified by HPLC (Waters Autopurification System, Phenomenex Luna C18 100 Angstroms). See the synthesis of compound **6** for a representative peptide synthesis (standard Fmoc chemistry on Wang resin).



compound 6

Compound 6. Compound 6 was assembled using standard Fmoc chemistry protocols on Fmoc-N-methylphenylalanine Wang resin and was acetylated on the N-terminus. Amino acids were coupled using coupling reagent 1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate (HATU) and base diisopropylethylamine (DIPEA). Peptide was cleaved off the solid support with trifluoroacetic acid: triisopropylsilane: water (95:2.5:2.5) for 1 hour at room temperature. Resin was filtered and filtrate was evaporated and peptide was precipitated with ethyl ether, centrifuged and ethyl ether was decanted off. Addition of ethyl ether, centrifugation and ether decantation was repeated twice and peptide pellet was allowed to dry. Crude peptide pellet were solubilized in dimethyl sulfoxide and purified by reverse phase chromatography on a C18 column using acetonitrile/water buffers. Purified fractions were analyzed by liquid chromatography mass spectrometry, pooled and lyophilized. LCMS (ES, *m/z*): $[M+H]^+$ 667.2

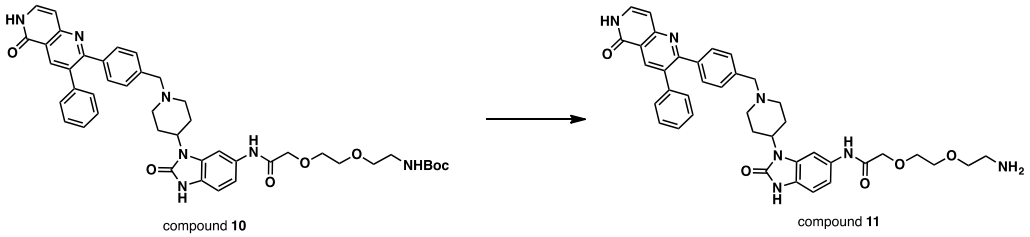
Compound 10. *tert*-butyl (2-(2-(2-oxo-2-((2-oxo-3-(1-(4-(5-oxo-3-phenyl-5,6-dihydro-1,6-naphthyridin-2-yl)benzyl)piperidin-4-yl)-2,3-dihydro-1*H*-benzo[*d*]imidazol-5-yl)amino)ethoxy)ethoxy)ethyl)carbamate.



Compound **9** was prepared similar to as described previously. [1] 2-[2-(2-[(tert-butoxy)carbonyl]aminoethoxy)ethoxy]acetic acid (410 mg, 1.56 mmol) was dissolved in DMA (30 mL) and treated sequentially with N,N-diisopropylethylamine (1.5 mL, 9.4 mmol), HATU (700 mg, 1.9 mmol) and compound **7** (1 g, 1.56 mmol). The reaction mixture was stirred for 2 h at room temperature, quenched by addition of water (100 mL) and then extracted with dichloromethane (3 x 100 mL). The combined organics were washed with water, dried over anhydrous sodium sulfate, and concentrated under vacuum. Purification by flash column chromatography (dichloromethane/methanol, 1:0-95:5) gave 0.80 g (66%) of tert-butyl N-[2-[2-([[2-oxo-3-(1-[[4-(5-oxo-3-phenyl-5,6-dihydro-1,6-naphthyridin-2-yl)phenyl]methyl]piperidin-4-yl)-2,3-dihydro-1H-1,3-benzodiazol-5-yl]carbamoyl]methoxy)ethoxy]ethyl]carbamate(compound **10**) as a light yellow solid.

^{1}H NMR (300 MHz, DMSO-d_6, *ppm*): δ 11.61 (s, 1H), 10.80 (s, 1H), 9.58 (s, 1H), 8.40 (s, 1H), 7.62 (s, 1H), 7.52-7.48(m, 1H), 7.36-7.24 (m, 10H), 6.92-6.89(d, J = 8.4 Hz, 1H), 6.81-6.80(m, 1H), 6.71-6.68 (d, J = 8.4 Hz, 1H), 4.14-4.09(m, 1H), 4.07(s, 2H), 3.68-3.60(m, 4H), 3.53(s, 2H), 3.46-3.39(m, 3H), 3.13-3.09(m, 2H), 2.96-2.93(m, 2H), 2.34-2.30(d, 2H), 2.12-2.08(m, 2H), 1.66-1.63(m, 2H), 1.35(s, 9H).

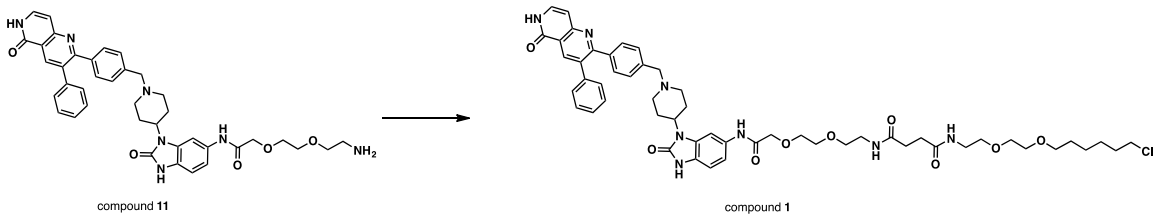
Compound 11. 2-(2-(2-aminoethoxy)ethoxy)-*N*-(2-oxo-3-(1-(4-(5-oxo-3-phenyl-5,6-dihydro-1,6-naphthyridin-2-yl)benzyl)piperidin-4-yl)-2,3-dihydro-1*H*-benzo[*d*]imidazol-5-yl)acetamide



Compound **10** (500 mg, 0.63 mmol) was dissolved in DCM (15 mL) and cooled in an ice/water bath. This mixture was treated, dropwise, with 4N HCl in 1,4-dioxane (7.5 mL). The reaction mixture was allowed to reach room temperature and stirred for 1 hr. The solid was collected by filtration and then dissolved in 10 mL of water. Addition of 5 mL of saturated aqueous sodium bicarbonate solution precipitated a solid that was collected by filtration, washed with water (3 x 10 mL) and then dried under vacuum to give 260 mg (59%) of compound **11** as an off-white solid.

LCMS (ES, *m/z*): $[M+H]^+$ = 688. ^{1}H NMR (300 MHz, DMSO-d_6, *ppm*): δ 10.09 (s, 1H), 9.61 (s, 1H), 8.38 (s, 1H), 7.61 (s, 1H), 7.53-7.50 (d, *J* = 7.2 Hz, 1H), 7.44-7.26 (m, 10H), 6.91-6.90 (d, *J* = 4.5 Hz, 1H), 6.84-6.81 (d, *J*

= 7.8 Hz, 1H), 4.07 (s, 3H), 3.69-3.67 (m, 2H), 3.64-3.60 (m, 2H), 3.53 (s, 2H), 3.42-3.38 (m, 4H), 2.95-2.92 (d, *J* = 9.6 Hz, 2H), 2.68-2.64 (t, *J* = 11.4 Hz, 2H), 2.33-2.20 (m, 2H), 2.12-2.04 (m, 2H), 1.65-1.62 (d, *J* = 10.2 Hz, 2H).



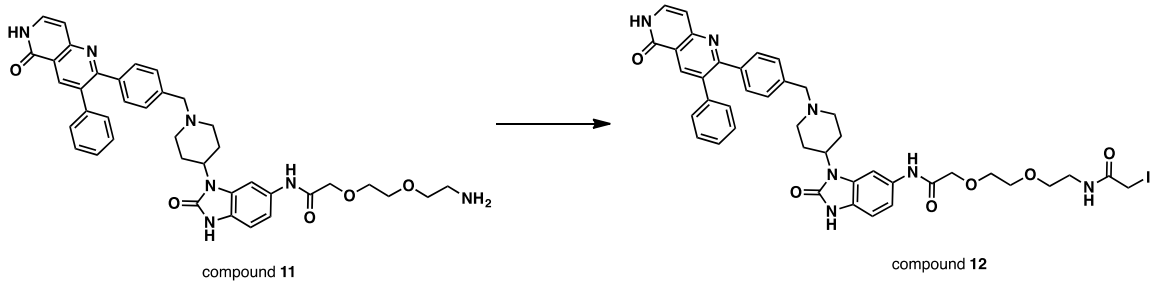
Compound 1. N^1-(2-(2-((6-chlorohexyl)oxy)ethoxy)ethyl)-N^4-(2-(2-(2-oxo-2-((2-oxo-3-(1-(4-(5-oxo-3-phenyl-5,6-dihydro-1,6-naphthyridin-2-yl)benzyl)piperidin-4-yl)-2,3-dihydro-1*H*-benzo[*d*]imidazol-5-yl)amino)ethoxy)ethoxy)ethyl)succinamide

Commercial 4-[2-[2-(6-chlorohexoxy)ethoxy]ethylamino]-4-oxo-butanoate (5 mg, 0.012 mmol) in 0.1 mL acetonitrile was added to an ice-bath cooled solution of 2-[2-(2-aminoethoxy)ethoxy]-N-[2-oxo-3-[1-[[4-(5-oxo-3-phenyl-6H-1,6-naphthyridin-2-yl)phenyl]methyl]-4-piperidyl]-1H-benzimidazol-5-yl]acetamide TFA salt (compound **7**, 9.0 mg, 0.012 mmol) in 0.1 mL of pH 8 borate buffer. The mixture was warmed to room temperature to stir for 2 hours. Concentration and purification by reverse-phase HPLC gave compound **1** (9.8 mg, 88% yield) as a colorless solid.

LC-MS: $[M+H]^+$ = 995. HRMS calc'd for $C_{53}H_{66}ClN_8O_9$ $(M+H)^+$ 993.4636, found: 993.4626

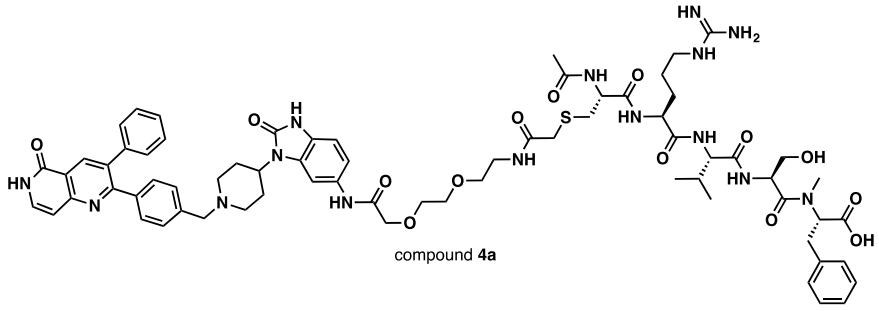
^{1}H NMR (500 MHz, DMSO-d_6) δ 11.6 (d, *J*=5.9 Hz, 1H), 10.81 (s, 1H), 9.61 (s, 1H), 8.39 (s, 1H), 7.91 (t, *J*=5.6 Hz, 1H), 7.81 (t, *J*=5.6 Hz, 1H), 7.62 (d, *J*=1.9 Hz, 1H), 7.50 (dd, *J*=7.3, 5.9 Hz, 1H), 7.36-7.30 (m, 5H), 7.29-7.23 (m, 5H), 6.9 (d, *J*=8.5 Hz, 1H), 6.69 (d, *J*=7.3 Hz, 1H), 4.07 (s, 2H), 3.68 (dd, *J*=5.9, 3.5 Hz, 2H), 3.63-3.58 (m, 4H), 3.45 (m, 6H), 3.35 (m, 5H), 3.20 (q, *J*=5.8 Hz, 2H), 3.16 (q, *J*=5.9 Hz, 2H), 2.98-2.90 (m, 2H), 2.73-2.42 (m, 8H), 2.09 (t, *J*=11.6 Hz, 2H), 1.73-1.58 (m, 4H), 1.50-1.41 (m, 2H), 1.40-1.32 (m, 2H), 1.32-1.20 (m, 2H).

Compound 12. 2-iodo-*N*-(2-(2-(2-oxo-2-((2-oxo-3-(1-(4-(5-oxo-3-phenyl-5,6-dihydro-1,6-naphthyridin-2-yl)benzyl)piperidin-4-yl)-2,3-dihydro-1*H*-benzo[*d*]imidazol-5-yl)amino)ethoxy)ethoxy)ethyl)acetamide

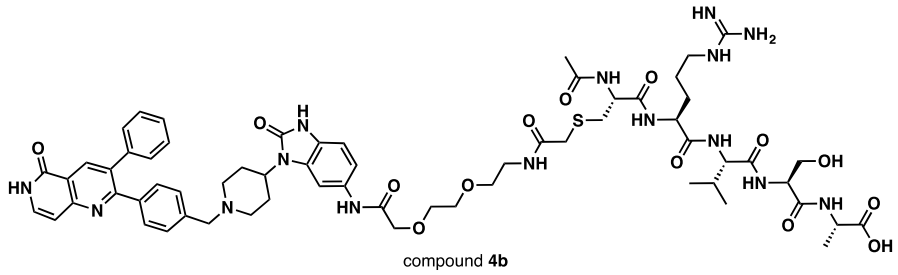


Compound **11** (90 mg, 0.13 mmol) was dissolved in DMF (3 mL) and the reaction mixture was treated with N,N-diisopropylethylamine (0.2 mL, 1.2 mmol) and cooled with an ice/water bath. The cool mixture was treated with 2,5-dioxopyrrolidin-1-yl 2-iodoacetate. After 10 min, the mixture was carefully concentrated and purified by reverse-phase HPLC (ACN/Water(0.05%NH_3) to give 29.8 mg (27%) of compound **12** as an off-white solid.

LCMS (ES, *m/z*): $[M+H]^+$ =856. ^{1}H NMR (300 MHz, CD_3OD, *ppm*): δ 8.60 (s, 1H), 7.70 (s, 1H), 7.50-7.47 (d, *J* = 6.6 Hz, 1H), 7.41-7.20 (m, 11H), 7.04-7.01 (d, *J* = 8.4 Hz, 1H), 6.88-6.86 (d, *J* = 7.5 Hz, 1H), 4.28-4.24 (m, 1H), 4.17 (s, 2H), 3.80-3.78 (m, 2H), 3.74-3.73 (m, 2H), 3.63-3.58 (m, 7H), 3.46-3.35 (m, 3H), 3.09-3.05 (d, *J* = 11.7 Hz, 2H), 2.54-2.42 (m, 2H), 2.28-2.24 (m, 2H), 1.79-1.75 (d, *J* = 11.1 Hz, 2H).

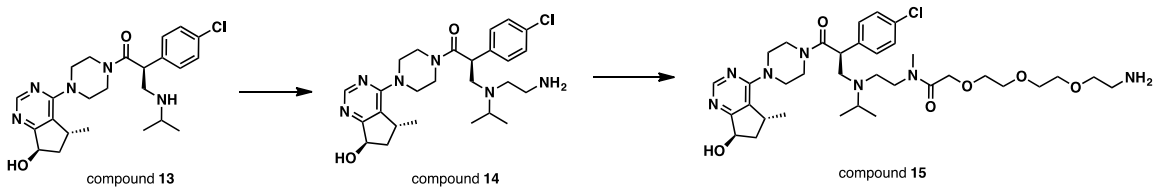

compound **4a**

Compound 4a. To a solution of 2-iodo-N-(2-(2-(2-oxo-2-((2-oxo-3-(1-(4-(5-oxo-3-phenyl-5,6-dihydro-1,6-naphthyridin-2-yl)benzyl)piperidin-4-yl)-2,3-dihydro-1H-benzo[d]imidazol-5-yl)amino)ethoxy)ethoxy)ethyl)acetamide (**12**, 6.0 mg, 0.0090 mmol) in 0.90 mL of pH8 HEPES, a solution of compound N-Ac-CRVSF(NMe) (7.7 mg, 0.0090 mmol) in 0.10 mL of DMSO was added. The reaction mixture was stirred at room temperature overnight, then concentrated. The residue was subjected to reverse phase HPLC (acetonitrile in water with 0.05% TFA, 5 to 50% in 30 min) to afford the title compound as a white solid (8.9 mg, 71%). LC-MS: $[M+2H]^{2+}$ = 697. HRMS calc'd for $C_{70}H_{88}N_{15}O_{14}S$ $(M+H)^+$ 1394.6350, found: 1394.6331.


compound **4b**

Compound 4b. To a solution of 2-iodo-*N*-(2-(2-(2-oxo-2-((2-oxo-3-(1-(4-(5-oxo-3-phenyl-5,6-dihydro-1,6-naphthyridin-2-yl)benzyl)piperidin-4-yl)-2,3-dihydro-1*H*-benzo[*d*]imidazol-5-yl)amino)ethoxy)ethoxy)ethyl)acetamide (**12**, 2.4 mg, 0.0041 mmol) in 0.90 mL of pH8 HEPES buffer, 1 M, a solution of N-Ac-CRVSA (3.5 mg, 0.0041 mmol) in 0.10 mL of DMSO was added. The resulting mixture was stirred at room temperature overnight, then directly loaded to reverse phase HPLC (acetonitrile in water with 0.1% TFA, 20 to 40% in 20 min) to afford the title compound as a white solid (**4b**, 4.4 mg, 82%). LC-MS: $[M+H]^+$ = 1305. HRMS calc'd for $C_{63}H_{88}N_{15}O_{14}S$ $(M+H)^+$ 1304.5881, found: 1304.5859.

Compound 15. 2-[2-[2-(2-aminoethoxy)ethoxy]ethoxy]-N-(2-[[(2S)-2-(4-chlorophenyl)-3-[4-[(5R,7R)-7-hydroxy-5-methyl-5H,6H,7H-cyclopenta[d]pyrimidin-4-yl]piperazin-1-yl]-3 oxopropyl](propan-2-yl)amino]ethyl)-N-methylacetamide



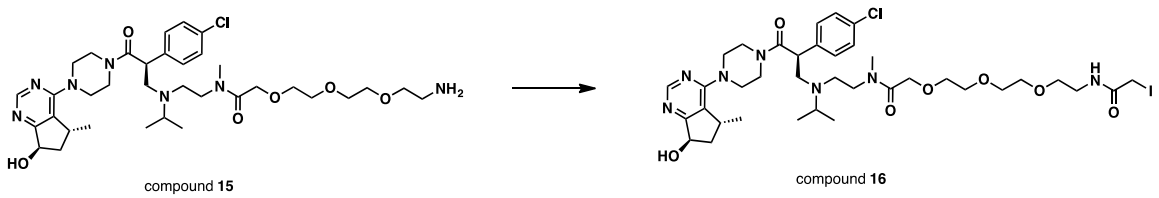
Compound **13** was prepared as described previously.[2] Compound **13** (500 mg, 1.09 mmol) and tert-butyl N-methyl-N-(2-oxoethyl)carbamate (566 mg, 3.27 mmol) were dissolved in 20 mL of 1,2-dichloroethane. The resulting solution was stirred for 0.5 h at room temperature. $NaBH(OAc)_3$ (691 mg, 3.27 mmol) was added in one portion. The resulting solution was stirred overnight at room temperature. The combination of five of the above reactions were concentrated under vacuum and the residue purified by flash column chromatography (dichloromethane/methanol, 1:0~20:1) to give 2.8 g (69%) of the reductive amination product as a light yellow solid. This material was dissolved in 50 mL of DCM and the mixture cooled in an ice/water bath. 4N HCl in 1,4-dioxane (10 mL) was added dropwise and the reaction mixture was allowed to reach room temperature for 3 hr. Concentration under vacuum gave ~2.8 (crude, HCl salt) of compound **14** as a yellow solid.

^{1}H NMR (300 MHz, DMSO-d_6, *ppm*) δ 8.45 (s, 1H), 7.58 (d, *J* = 50.8 Hz, 1H), 7.41 (s, 4H), 5.47-5.35 (m, 1H), 4.85 (t, *J* = 6.5 Hz, 1H), 4.37 (d, *J* = 8.1 Hz, 1H), 3.55 (dt, *J* = 63.2, 36.9 Hz, 11H), 3.04 (d, *J* = 11.6 Hz, 2H), 2.92-2.84 (m, 1H), 2.67 (dd, *J* = 22.8, 11.2 Hz, 2H), 2.48-2.38 (m, 1H), 2.27 (d, *J* = 34.1 Hz, 1H), 1.95 (h, *J* = 8.0, 6.7 Hz, 2H), 1.05 (d, *J* = 6.8 Hz, 3H), 0.96 (d, *J* = 6.6 Hz, 3H), 0.85 (d, *J* = 6.3 Hz, 3H).

2-[2-[2-(2-[[(tert-butoxy)carbonyl]amino]ethoxy)ethoxy]ethoxy]acetic acid (260 mg, 0.85 mmol) was dissolved in THF (20 mL) and the mixture was treated sequentially with HATU (354 mg, 0.93 mmol) and N,N-diisopropylethylamine (3.4 mmol). After 5 min stirring at room temperature, compound **14** (560 mg, 1.09 mmol) was added. The solution was stirred for 1 hour. The combination of five of the above reactions were diluted with 50 mL of water and extracted with dichloromethane (3 x 50 mL). The organic was washed with brine (3 x 50 mL), dried over sodium sulfate, concentrated under vacuum and purified by flash column chromatography (DCM/MeOH, 1:0 – 20:1) to give 2.3 g (44%) of tert-butyl (S)-17-(4-chlorophenyl)-18-(4-((5R,7R)-7-hydroxy-5-methyl-6,7-dihydro-5H-cyclopenta[d]pyrimidin-4-yl)piperazin-1-yl)-15-isopropyl-12-methyl-11,18-dioxo-3,6,9-trioxa-12,15-diazaoctadecylcarbamate as an off-white solid. A solution of a portion of this material (2.0 g, 2.49 mmol) in 60 mL dichloromethane was cooled in an ice/water bath. 4N HCl in 1,4-dioxane (20 mL) was added dropwise. The mixture was allowed to warm to room temperature and stirred for one hour. Concentration gave 1.82 g (83%) of compound **15** as a light yellow solid.

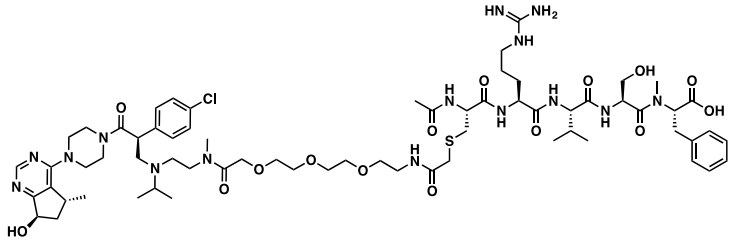
LCMS (ES, *m/z*): $[M+H-5HCl]^+$ =704. ^{1}H NMR (300 MHz, CD_3OD, *ppm*): δ8.59 (s, 1H), 7.46 (s, 4H), 5.34-5.29 (t, *J* = 15.9 Hz, 1H), 4.97-4.96 (m, 1H), 4.78 (s, 1H), 4.35 (s, 2H), 3.99-3.82 (m, 8H), 3.80-3.66 (m, 14H), 3.59-3.45 (m, 3H), 3.17-3.14 (m, 2H), 3.10-3.07 (m, 3H), 2.30-2.27 (m, 1H), 2.20-2.14 (m, 1H), 1.54-1.52 (d, *J* = 6.3 Hz, 2H), 1.42-1.37 (m, 2H), 1.34-1.29 (m, 2H), 1.18-1.17 (m, 3H).

Compound 16. *N*-(2-(((*S*)-2-(4-chlorophenyl)-3-(4-((5*R*,7*R*)-7-hydroxy-5-methyl-6,7-dihydro-5*H*-cyclopenta[*d*]pyrimidin-4-yl)piperazin-1-yl)-3-oxopropyl)(isopropyl)amino)ethyl)-2-(2-(2-(2-iodoacetamido)ethoxy)ethoxy)ethoxy)-*N*-methylacetamide

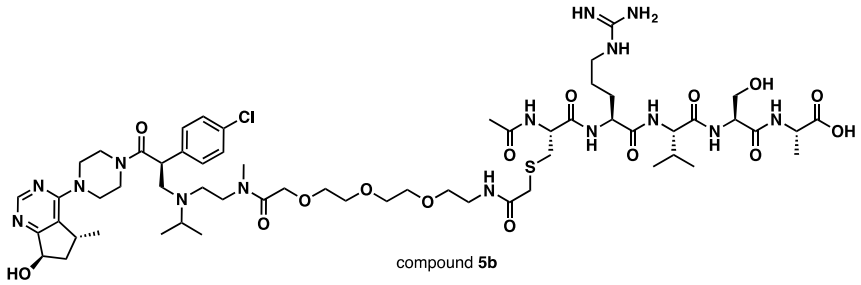


Compound **15** (130 mg, 0.15 mmoL) was dissolved in DMF (2.5 mL) and treated with N,N-diisopropylethylamine (0.25 mL, 1.5 mmol). The reaction mixture was cooled in an ice/water bath and treated with 2,5-dioxopyrrolidin-1-yl 2-iodoacetate (42 mg, 0.15 mmol). After 10 min, the mixture was purified by reverse phase HPLC (ACN/water(0.05% NH_3) to give 27 mg (21%) of compound **16** as an off-white solid.

LCMS (ES, *m/z*): $[M+H]^+$ =872. ^1H NMR (300 MHz, CD_3OD, *ppm*): δ 8.42-8.41 (m, 1H), 7.38-7.32 (m, 4H), 5.00-4.95 (m, 1H), 4.59 (s, 1H), 4.28-4.25 (m, 1H), 4.24 (s, 2H), 4.04-3.99 (m, 1H), 3.86-3.82 (m, 2H), 3.70-3.62 (m, 14H), 3.56-3.51 (m, 3H), 3.42 (s, 1H), 3.38-3.32 (m, 2H), 3.26-3.19 (m, 2H), 2.95-2.91 (m, 4H), 2.70-2.58 (m, 3H), 2.11-2.09 (m, 2H), 1.14-1.11 (d, *J* = 6.9 Hz, 3H), 1.05-0.97 (m, 3H), 0.93-0.89 (t, *J* = 12.9 Hz, 3H).



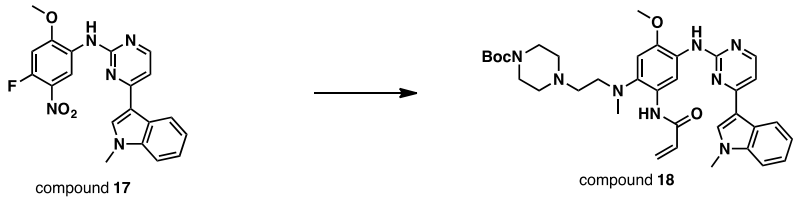
Compound 5a. To a solution of N-(2-(((S)-2-(4-chlorophenyl)-3-(4-((5R,7R)-7-hydroxy-5-methyl-6,7-dihydro-5H-cyclopenta[d]pyrimidin-4-yl)piperazin-1-yl)-3-oxopropyl)(isopropyl)amino)ethyl)-2-(2-(2-(2-(2-iodoacetamido)ethoxy)ethoxy)ethoxy)-N-methylacetamide (**15**, 2.4 mg, 0.0036 mmol) in 450 uL of pH8 HEPES buffer, 1 M, a solution of N-Ac-CRVSF(NMe) (3.1 mg, 0.0036 mmol) in 100 uL of DMSO was added. The reaction mixture was stirred at room temperature overnight, then concentrated *in vacuo*. The residue was subjected to reverse phase HPLC (acetonitrile in water with 0.1% TFA, 20 to 40% in 20 min) to afford the title compound as a white solid (**5a**, 1.9 mg, 38%). LC-MS: $[M+H]^+$ = 1411. HRMS calc'd for $C_{66}H_{101}ClN_{15}O_{15}S$ $(M+H)^+$ 1410.7005, found: 1410.6996.



Compound 5b. To a solution of *N*-(2-(((*S*)-2-(4-chlorophenyl)-3-(4-((5*R*,7*R*)-7-hydroxy-5-methyl-6,7-dihydro-5*H*-cyclopenta[*d*]pyrimidin-4-yl)piperazin-1-yl)-3-oxopropyl)(isopropyl)amino)ethyl)-2-(2-(2-(2-(2-iodoacetamido)ethoxy)ethoxy)ethoxy)-*N*-methylacetamide (**15**, 7.3 mg, 0.0126 mmol) in 450 uL of pH8 HEPES buffer, 1 M, the compound N-Ac-CRVSA (11.1 mg, 0.0126 mmol) solution in 100 uL of DMSO was added. The resulting mixture was stirred at room temperature overnight, then directly loaded to reverse phase HPLC

(acetonitrile in water with 0.1% TFA, 20 to 40% in 20 min) to afford the title compound as a white solid (**5b**, 10.4 mg, 62%). LC-MS: $[M+H]^+$ = 1321. HRMS calc'd for $C_{59}H_{95}ClN_{15}O_{15}S$ $(M+H)^+$ 1330.6463, found: 1320.6533

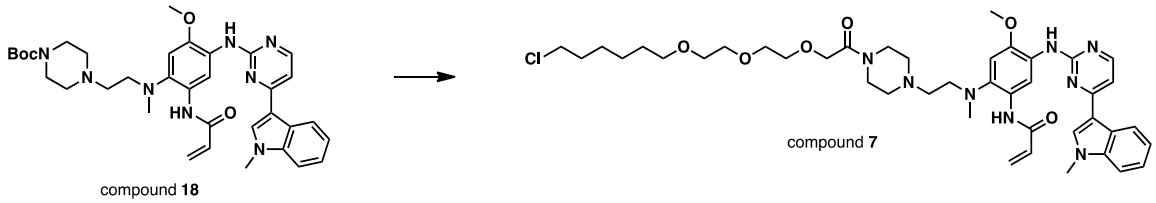
Compound 7. *N*-(2-((2-(4-(2-(2-(2-((6-chlorohexyl)oxy)ethoxy)ethoxy)acetyl)piperazin-1-yl)ethyl)(methyl)amino)-4-methoxy-5-((4-(1-methyl-1*H*-indol-3-yl)pyrimidin-2-yl)amino)phenyl)acrylamide.



tert-butyl 4-(2-(methylamino)ethyl)piperazine-1-carboxylate (4.6 g, 19.1 mmol) was added to a suspension of compound **17** (7.5 g, 19.1 mmol) and DIPEA (9.9 mL, 57.3 mmol) in 2,2,2-trifluoroethanol (100 mL) – [compound **17** is commercially available]. The resulting mixture was stirred at 105 °C for 2 h. The mixture was cooled to room temperature, concentrated and purified by flash column chromatography (0-4% 7N NH_3/MeOH in DCM) to give an orange solid (7.2 g, yield: 61%). [LCMS (ESI) *m/z*: 617.3 $(M + H)^+$; HRMS calc'd for $C_{32}H_{41}N_8O_5$ $(M+H)^+$ 617.3122, found: 617.3194].

This solid was dissolved in ethanol (90 mL) and the solution treated with iron (3.93 g, 70.2 mmol), and ammonium chloride (0.44 g, 8.19 mmol). The mixture was heated at reflux for 3 hrs, cooled to room temperature and concentrated. Purification by flash column chromatography (0-5% 7N NH_3/MeOH in DCM) to give the aniline as a light yellow solid (5.9 g, 86%) [MS (ESI) *m/z*: 587.4 $(M + H)^+$]. This solid was dissolved in THF (20 mL) and sat. aqueous $NaHCO_3$ (20 mL) and the mixture cooled in an ice/water bath. A solution of acryloyl chloride (909 mg, 10.1 mmol) in THF (3 mL) was added dropwise. After 90 min, the mixture was diluted with ethyl acetate and the extracted organic layer dried over $MgSO_4$, filtered and concentrated onto silica. Purification by flash column chromatography (0–4% 7 N NH_3/MeOH in DCM) gave compound **18** (2.6 g, 41%).

LCMS (ESI) m/z: 641.3 $(M + H)^+$. ^1H NMR (400 MHz, DMSO) δ 9.36 (s, 1H), 8.97 (s, 1H), 8.62 (s, 1H), 8.32 (d, *J* = 5.3 Hz, 1H), 8.26 (d, *J* = 7.9 Hz, 1H), 7.90 (s, 1H), 7.52 (d, *J* = 8.1 Hz, 1H), 7.23-7.14 (m, 3H), 6.99 (s, 1H), 6.64 (dd, *J* = 16.9, 10.2 Hz, 1H), 6.25 (m, 1H), 5.77 (m, 1H), 3.91 (s, 3H), 3.86 (s, 3H), 3.28 (s, 4H), 3.00 (t, *J* = 6.4 Hz, 2H), 2.70 (s, 3H), 2.39 (t, *J* = 6.4 Hz, 2H), 2.30 (s, 4H), 1.38 (s, 9H).



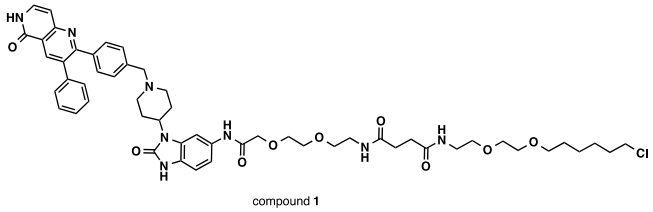
Compound **18** (200 mg, 0.31 mmol) in 1.6 mL 1,4-dioxane was treated with conc. HCl (1.6 mL, 36% in water) at room temperature. The reaction mixture was stirred for 30 min, diluted with 10 mL methanol and the solid collected by filtration and dried under vacuum (177 mg of a yellow solid). [LCMS (ESI) $[M+H]^+$=541]. The crude solid (4o mg, 0.069 mmol), 2-(2-[(6-chlorohexyl)oxy]ethoxyethoxy)acetic acid[3] (25 mg, 0.089 mmol), HATU (42 mg, 0.11 mmol) and DIPEA (33.5 mg, 0.259 mmol) were diluted with DMA (2 mL) and the reaction mixture

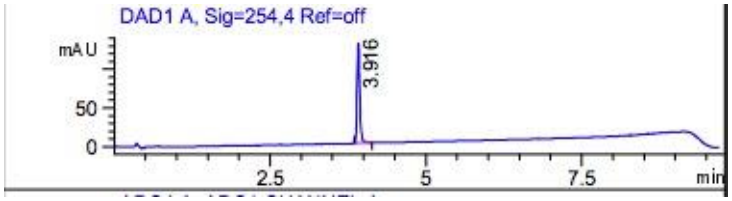
stirred for 30 min at room temperature. The solution was purified directly by reverse-phase HPLC (ACN:water 0.05% NH_3) to give compound **7** (12.3 mg, 21%).

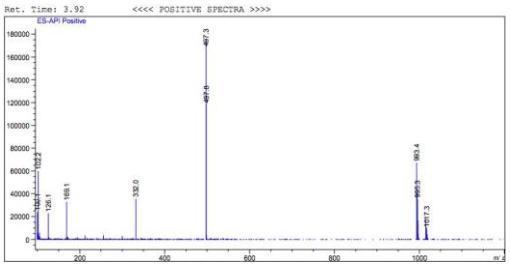
LCMS (ESI): $[M+H]^+$=805. ^{1}H NMR (300 MHz, DMSO-*d6*): δ 9.37 (s, 1H), 8.97 (s, 1H), 8.63 (s, 1H), 8.32 (d, *J* = 5.3 Hz, 1H), 8.25 (d, *J* = 7.9 Hz, 1H), 7.89 (s, 1H), 7.52 (d, *J* = 8.2 Hz, 1H), 7.29-7.10 (m, 3H), 6.99 (s, 1H), 6.64 (dd, *J* = 17.0, 10.2 Hz, 1H), 6.25 (dd, *J* = 17.0, 2.0 Hz, 1H), 5.76 (dd, *J* = 10.2, 2.0 Hz, 1H), 4.10 (s, 2H), 3.90 (s, 3H), 3.86 (s, 3H), 3.58 (t, *J* = 6.6 Hz, 2H), 3.55 – 3.34 (m, 14H), 3.00 (t, *J* = 6.4 Hz, 2H), 2.70 (s, 3H), 2.45-2.30 (m, 6H), 1.69-1.64 (m, 2H), 1.47-1.43 (m, 2H), 1.34-1.26 (m, 4H).

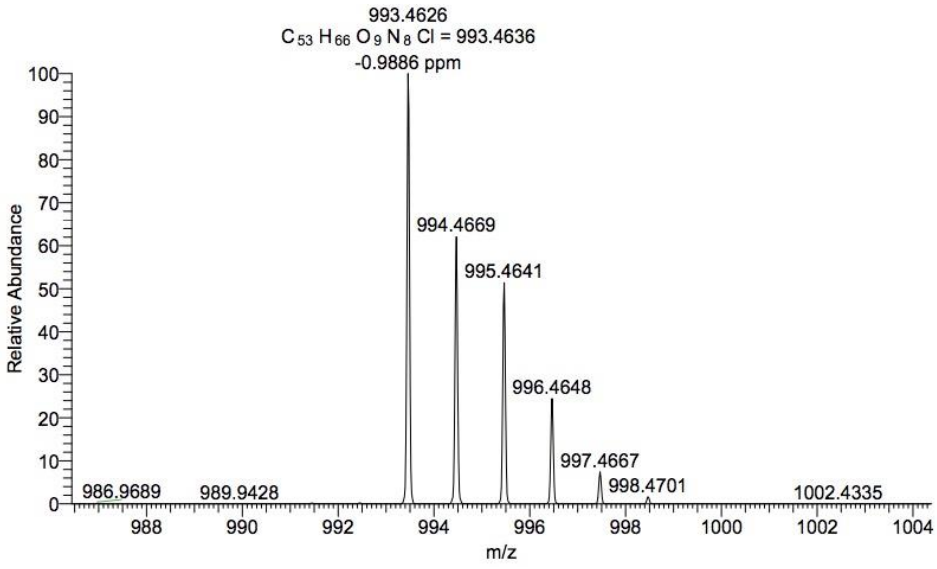
Images of spectra for key compounds

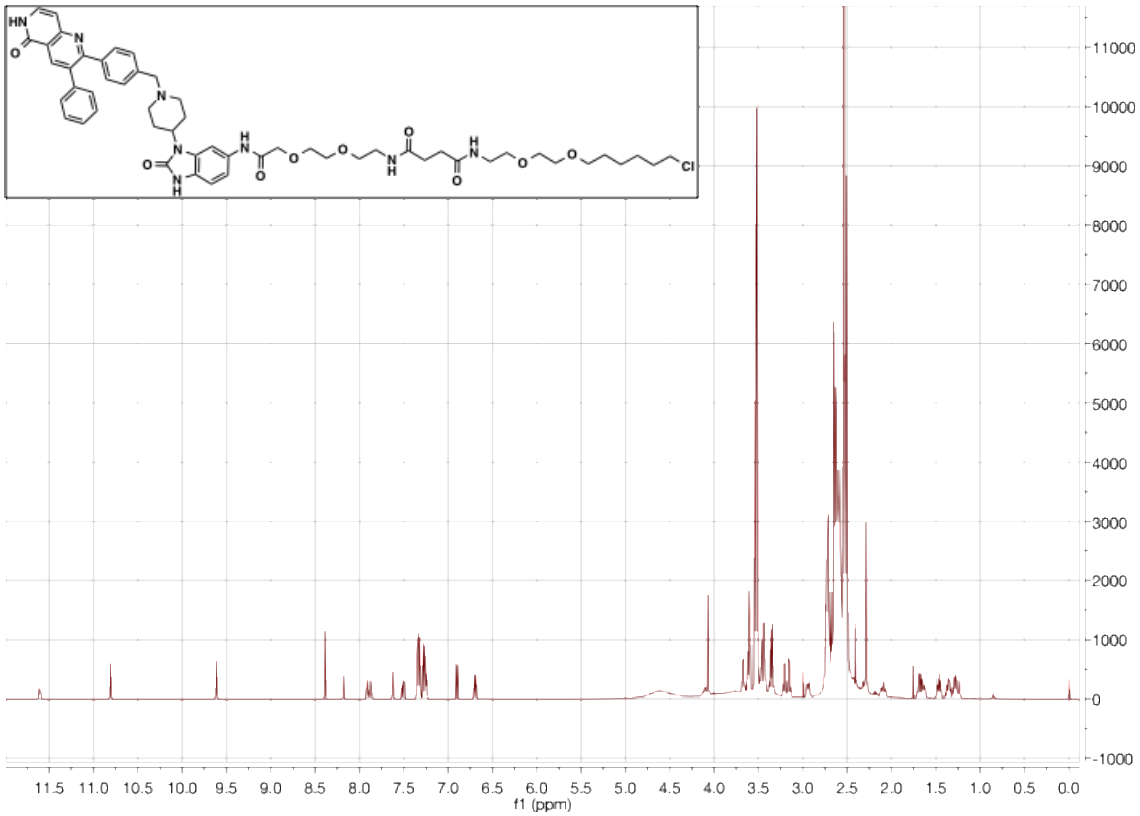
Compound 1:







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4000
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1000
0
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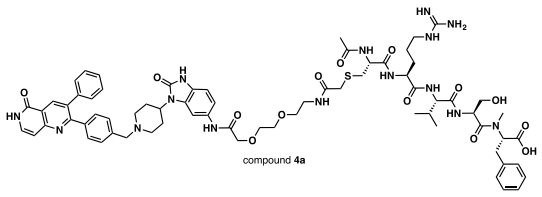
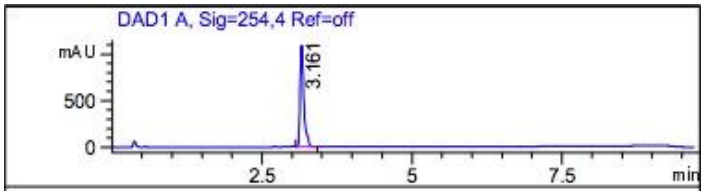
f1 (ppm)

Compound 4a:


compound 4a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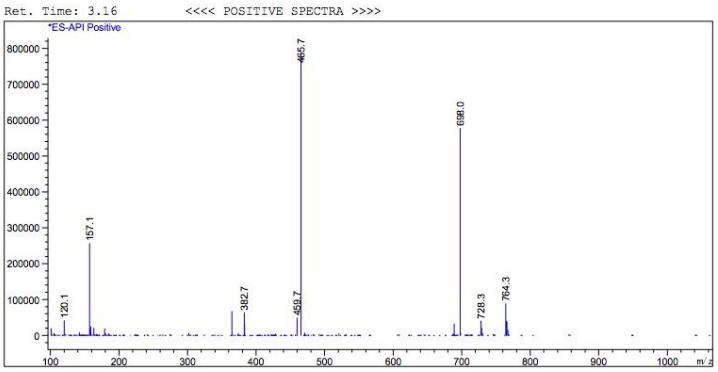
5
7.5
min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120.1
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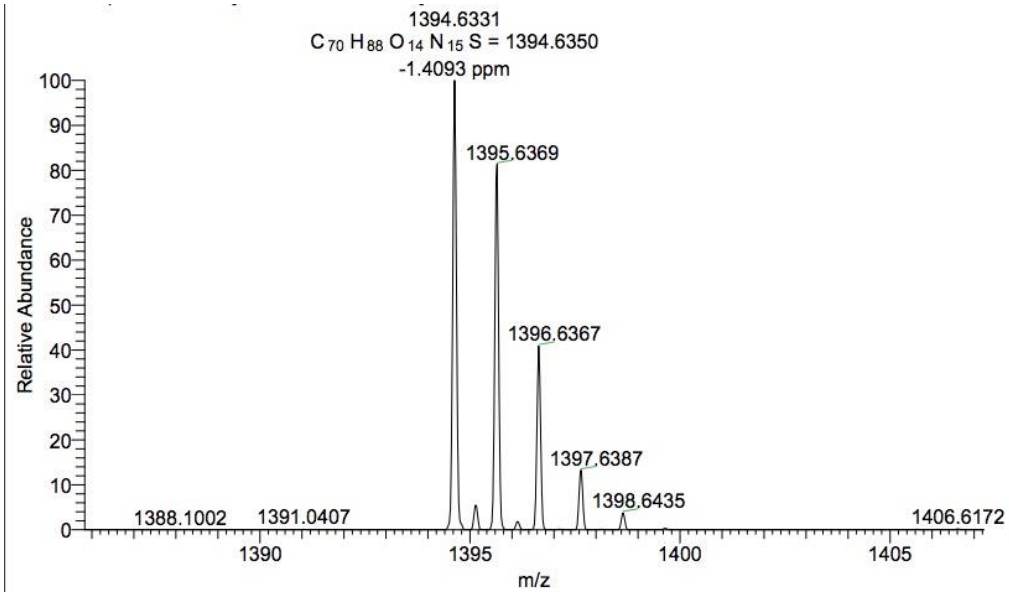
459.7
729.3
764.3
m/z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-1.4093 ppm
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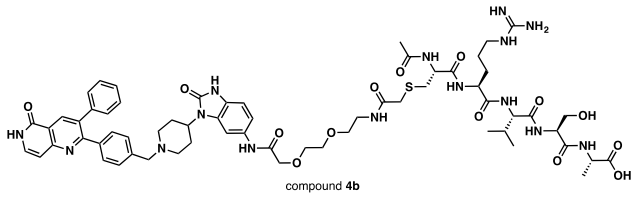
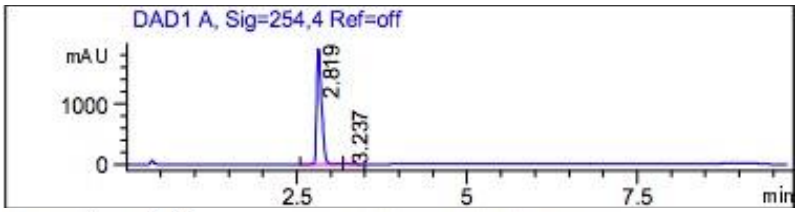
Relative Abundance
m/z

Compound 4b:


compound 4b



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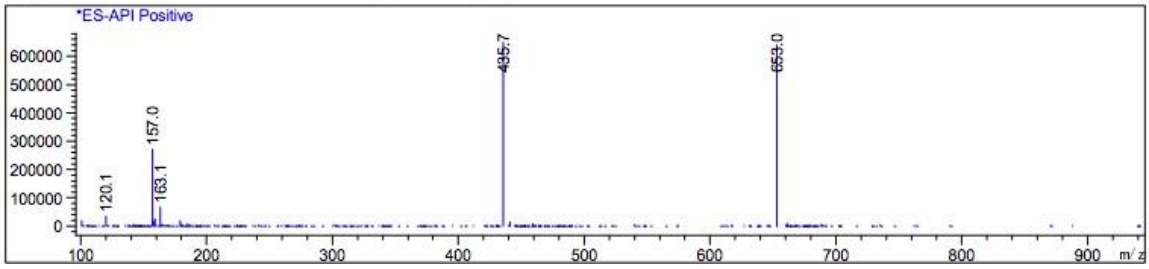
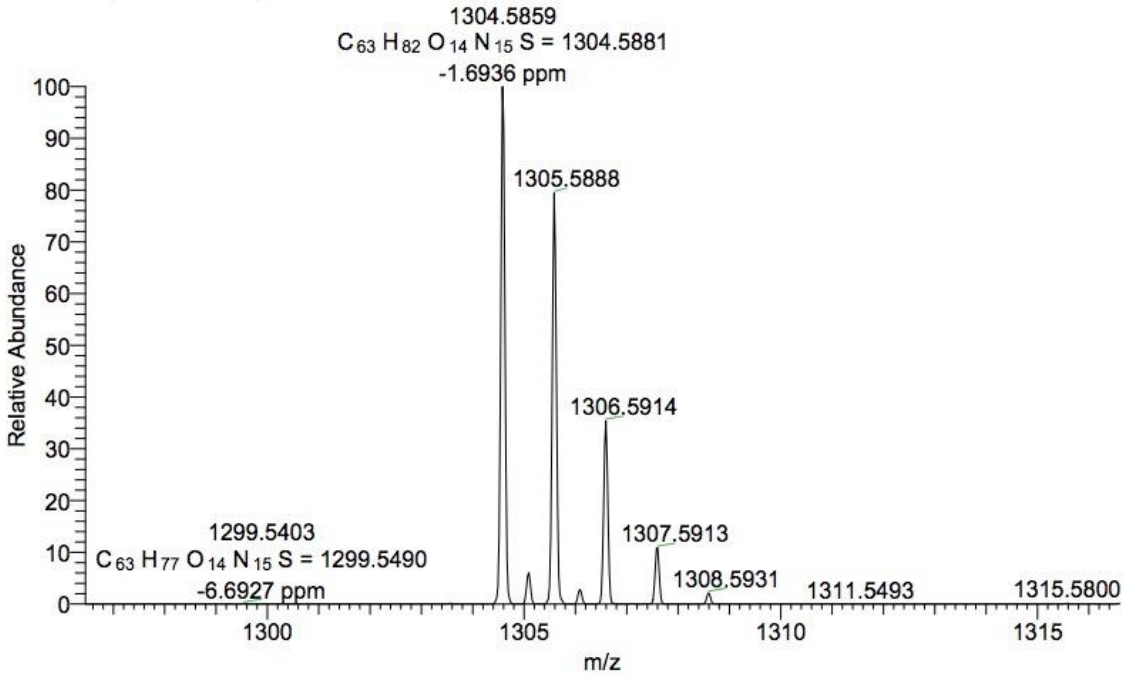
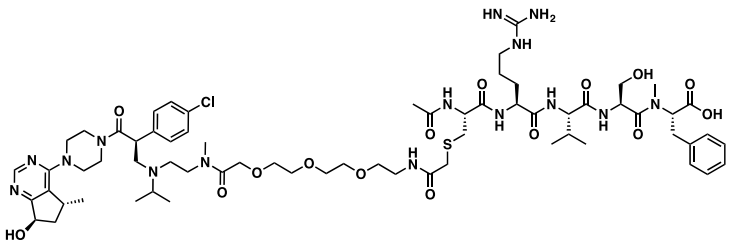
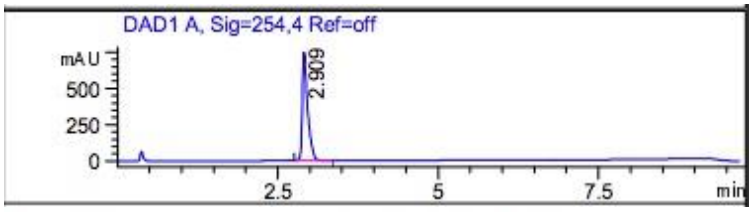




Compound 5a:





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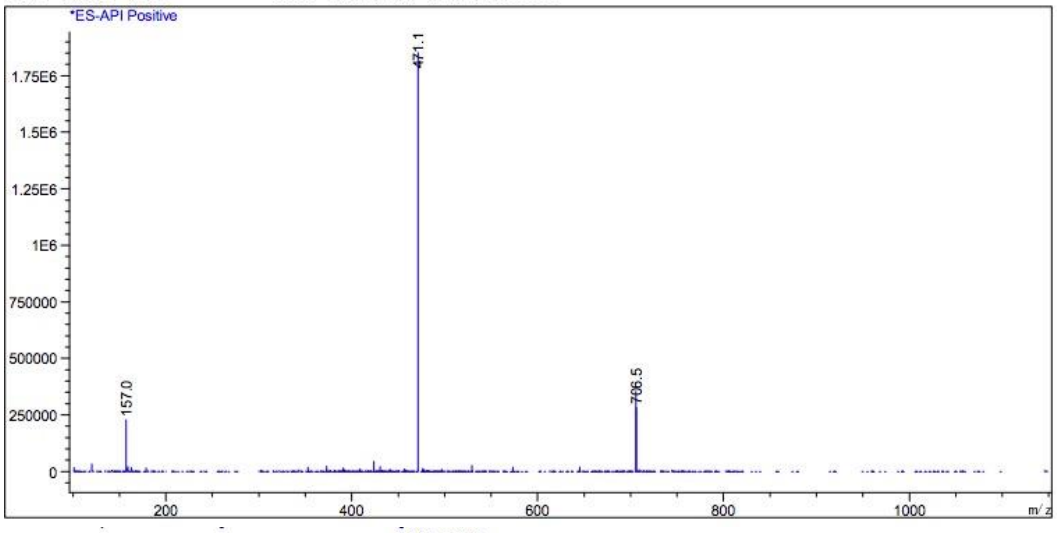
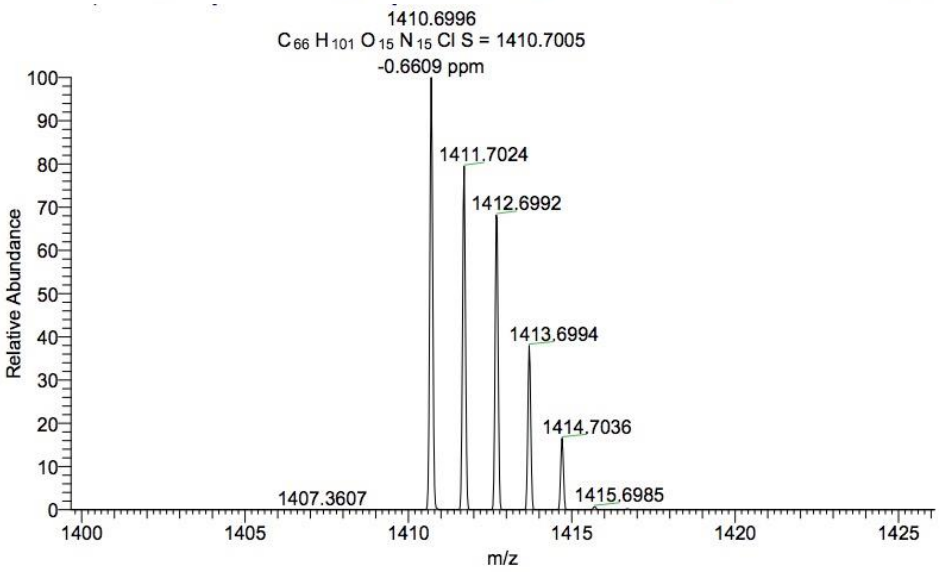
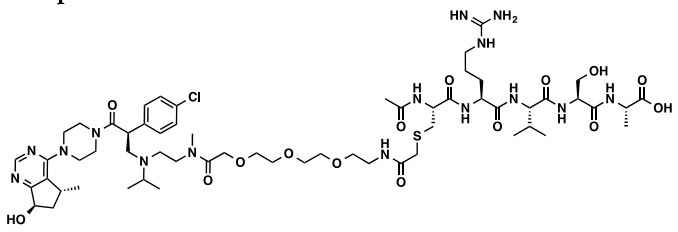
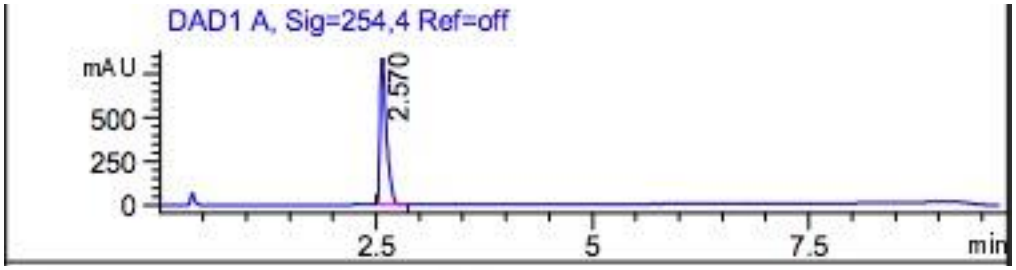




Compound 5b:





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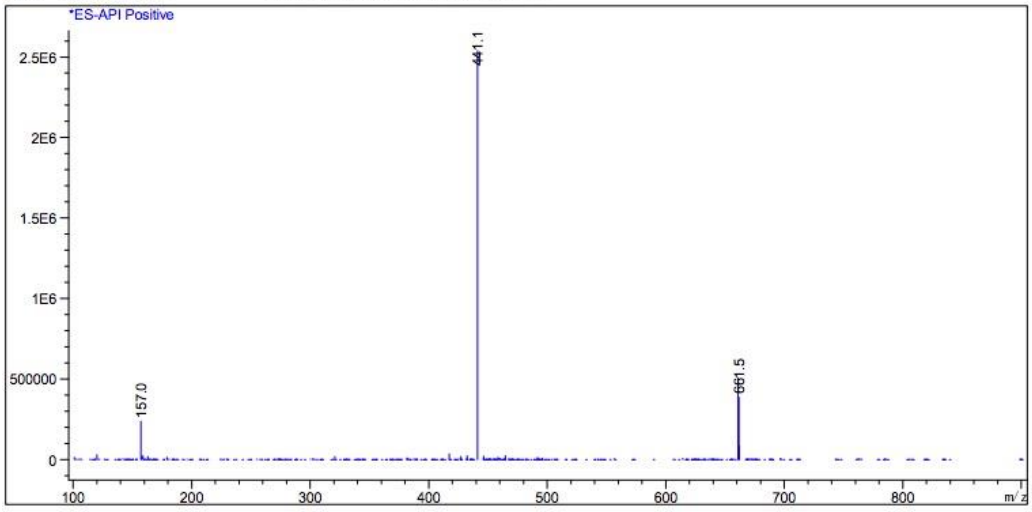

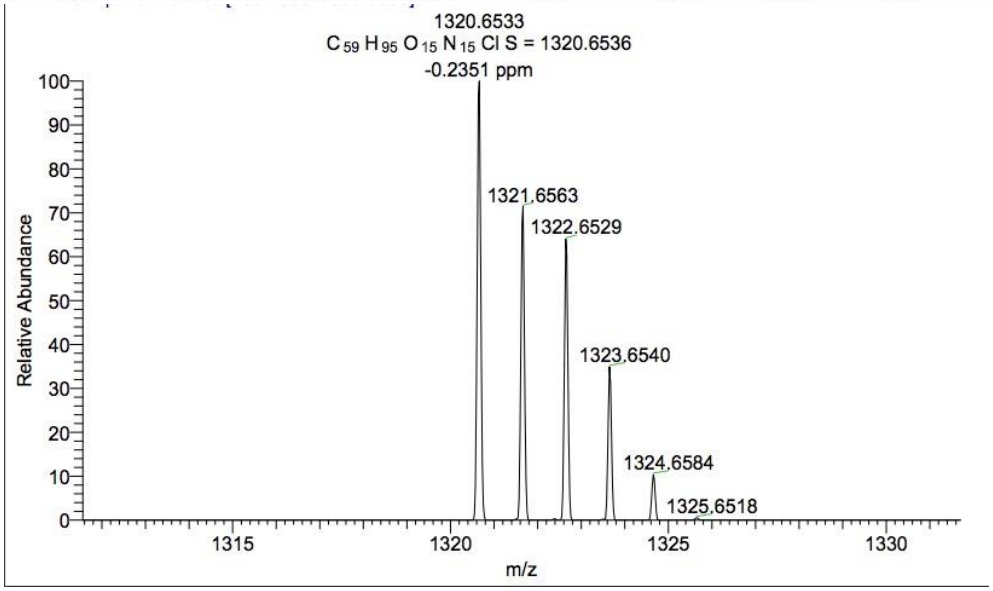

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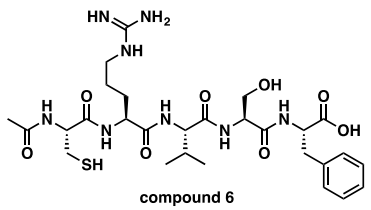

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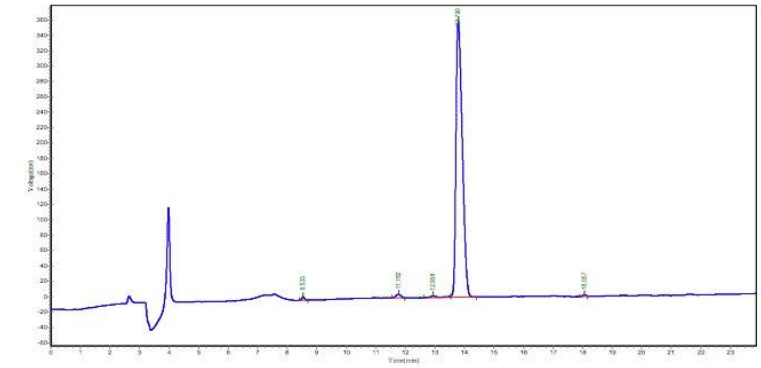

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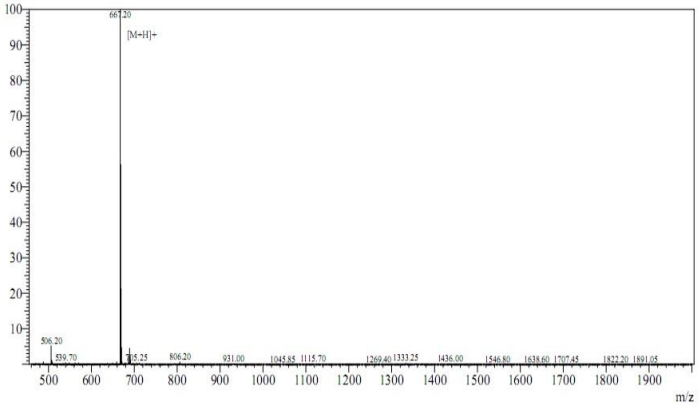
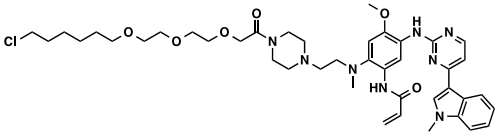
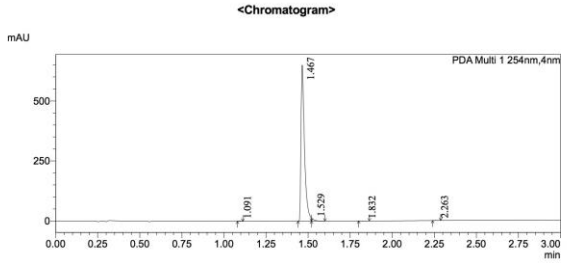
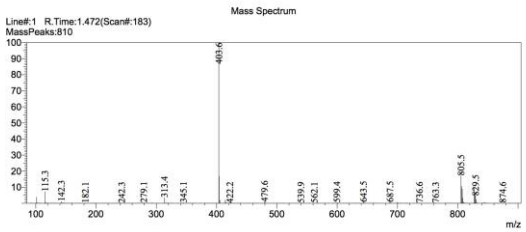
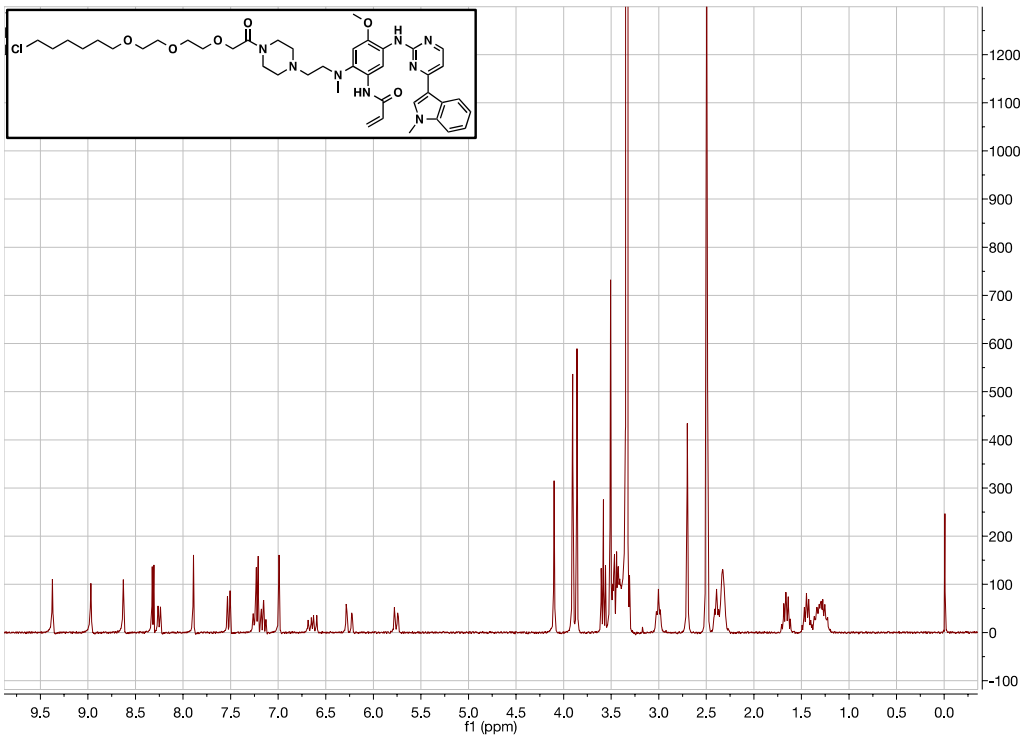


Compound 7:





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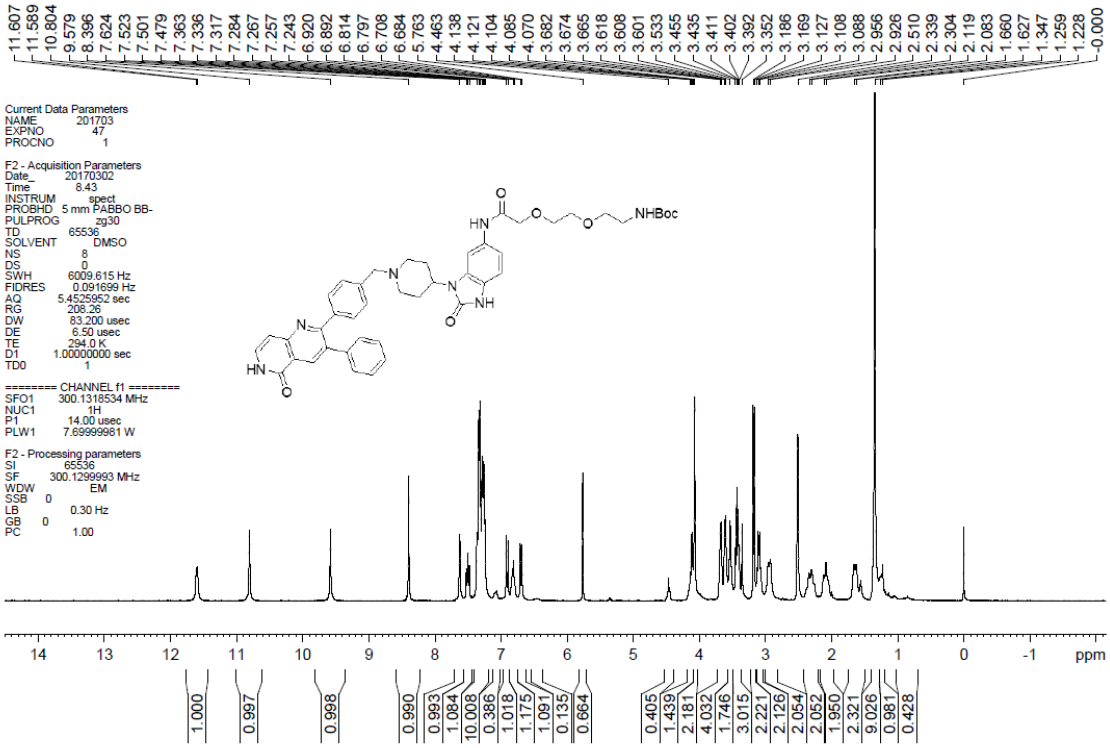
Compound 10: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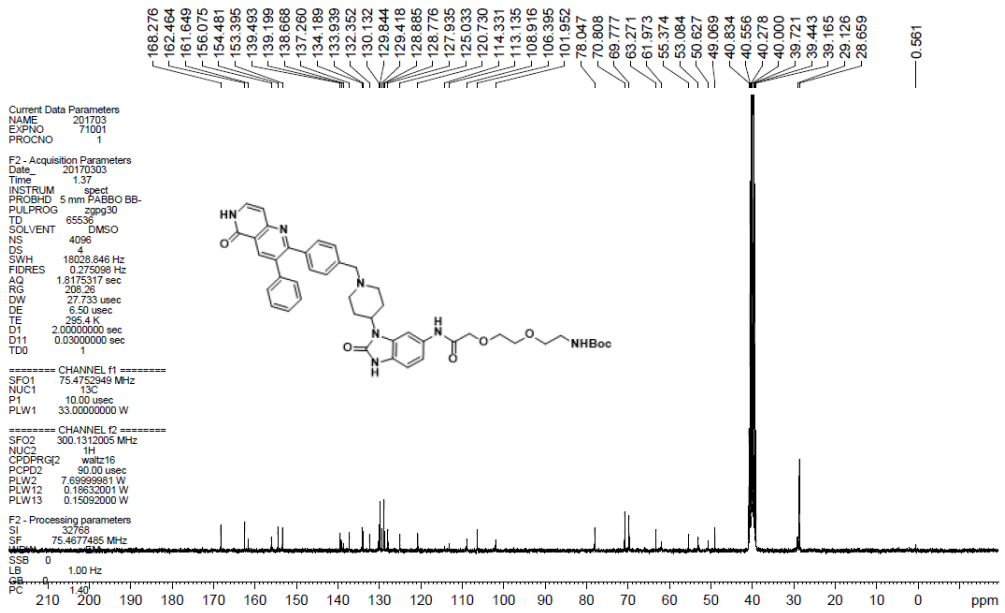
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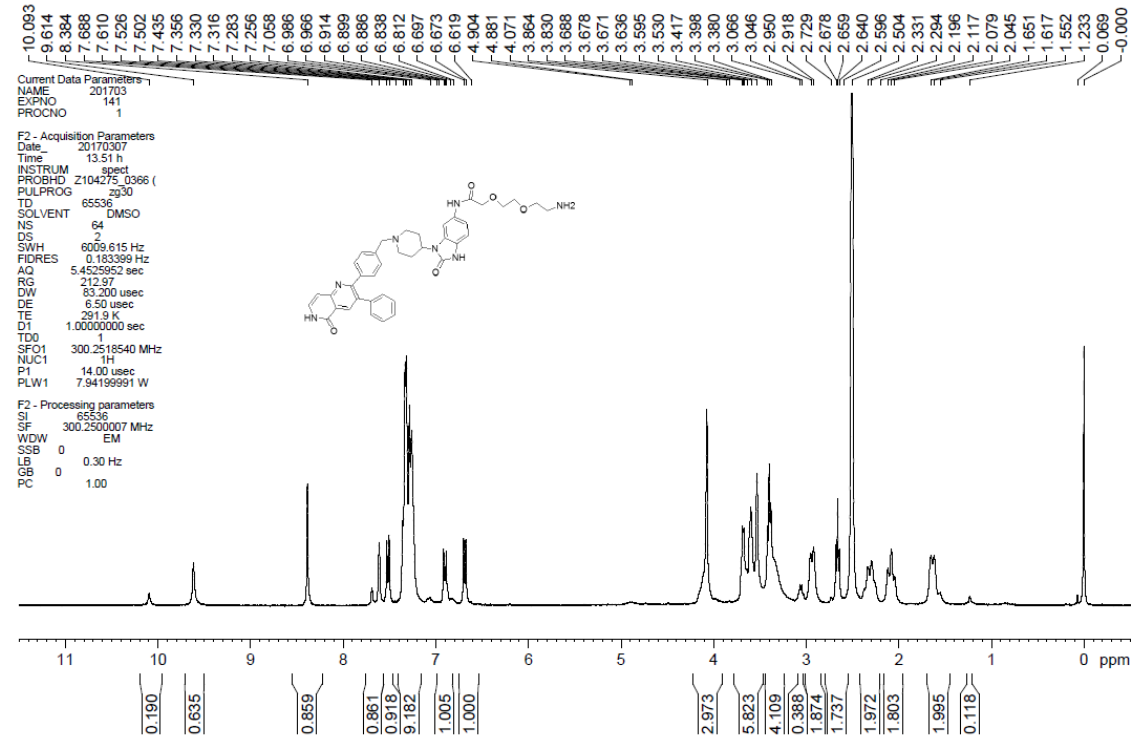
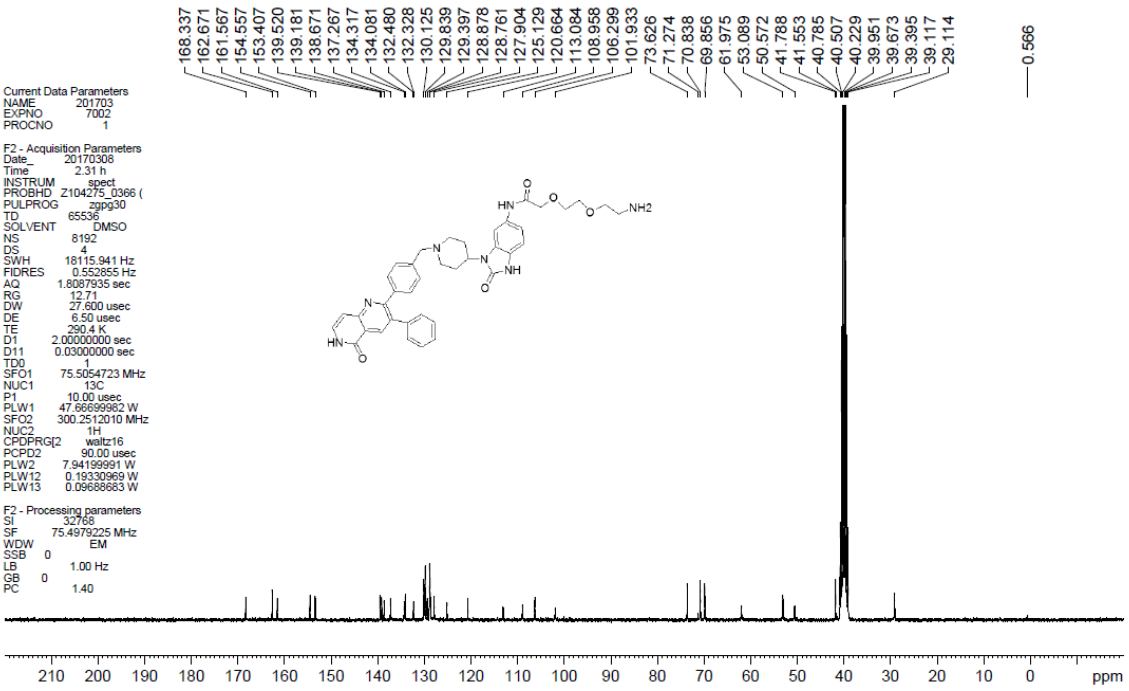
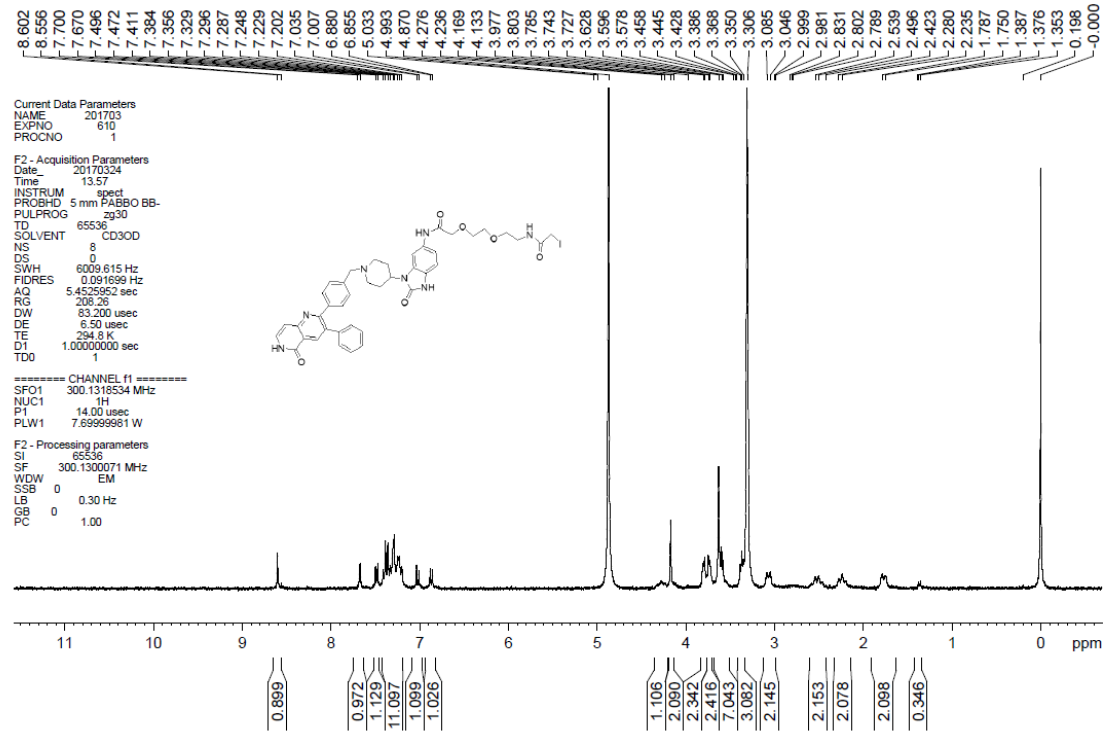
39.165
29.126
28.659
0.561
Current Data Parameters
NAME 201703
EXPNO 71001
PROCNO 1
F2 - Acquisition Parameters
Date_ 20170303
Time 1.37
INSTRUM spect
PROBHD 5 mm PABBO BB-
PULPROG zgpg30
TD 65536
SOLVENT DMSO
NS 4096
DS 4
SWH 18028.846 Hz
FIDRES 0.275098 Hz
AQ 1.8175317 sec
RG 208.26
DW 27.733 usec
DE 6.50 usec
TE 295.4 K
D1 2.00000000 sec
D11 0.03000000 sec
TD0 1
======== CHANNEL f1 ========
SFO1 75.4752949 MHz
NUC1 13C
P1 10.00 usec
PLW1 33.00000000 W
======== CHANNEL f2 ========
SFO2 300.1312005 MHz
NUC2 1H
CPDPRG[2 waltz16
PCPD2 90.00 usec
PLW2 7.69999981 W
PLW12 0.18632001 W
PLW13 0.15092000 W
F2 - Processing parameters
SI 32768
SF 75.4677485 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.40
HN
N
N
N
H
N
H
O
O
O
O
O
NHBoc
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 ppm

Compound 11:

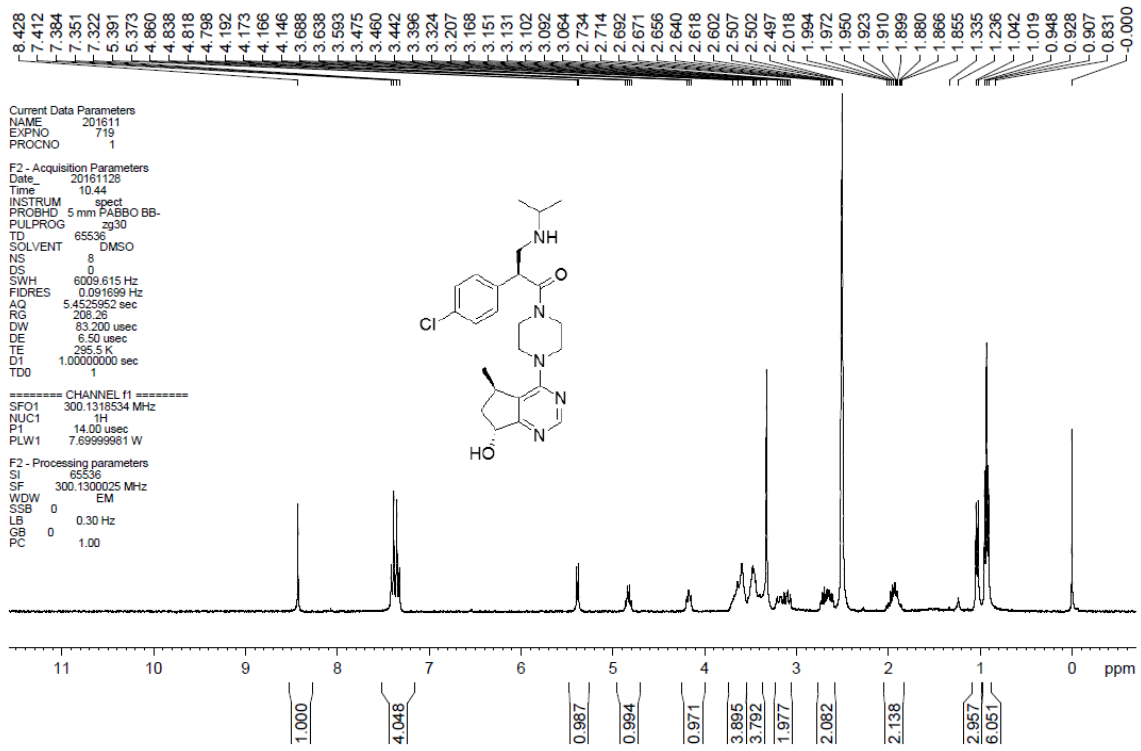

10.093
9.614
8.384
7.688
7.610
7.526
7.502
7.435
7.356
7.330
7.316
7.283
7.256
7.058
6.986
6.966
6.914
6.899
6.886
6.838
6.812
6.697
6.673
6.619
4.904
4.881
4.071
3.864
3.830
3.688
3.678
3.671
3.636
3.595
3.530
3.417
3.398
3.380
3.066
3.046
2.950
2.918
2.729
2.678
2.659
2.640
2.596
2.504
2.331
2.294
2.196
2.117
2.079
2.045
1.651
1.617
1.552
1.233
0.069
-0.000
Current Data Parameters
NAME 201703
EXPNO 141
PROCNO 1
F2 - Acquisition Parameters
Date_ 20170307
Time 13.51 h
INSTRUM spect
PROBHD Z104275_0366 (
PULPROG zg30
TD 65536
SOLVENT DMSO
NS 64
DS 2
SWH 6009.615 Hz
FIDRES 0.183399 Hz
AQ 5.4525952 sec
RG 212.97
DW 83.200 usec
DE 6.50 usec
TE 291.9 K
D1 1.00000000 sec
TD0 1
SFO1 300.2518540 MHz
NUC1 1H
P1 14.00 usec
PLW1 7.94199991 W
F2 - Processing parameters
SI 65536
SF 300.2500007 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00
HN
O
O
O
NH2
N
N
NH
O
HN
O
11 10 9 8 7 6 5 4 3 2 1 0 ppm
0.190
0.635
0.859
0.861
0.918
9.182
1.005
1.000
2.973
5.823
4.109
0.388
1.874
1.737
1.972
1.803
1.995
0.118


168.337
162.671
161.567
154.557
153.407
139.520
139.181
138.671
137.267
134.317
134.081
132.480
132.328
130.125
129.839
129.397
128.878
128.761
127.904
125.129
120.664
113.084
108.958
106.299
101.933
73.626
71.274
70.838
69.856
61.975
53.089
50.572
41.788
41.553
40.785
40.507
40.229
39.951
39.673
39.395
39.117
29.114
0.566
Current Data Parameters
NAME 201703
EXPNO 7002
PROCNO 1
F2 - Acquisition Parameters
Date_ 20170308
Time 2.31 h
INSTRUM spect
PROBHD Z104275_0366 (
PULPROG zgpg30
TD 65536
SOLVENT DMSO
NS 8192
DS 4
SWH 18115.941 Hz
FIDRES 0.552855 Hz
AQ 1.8087935 sec
RG 12.71
DW 27.600 usec
DE 6.50 usec
TE 290.4 K
D1 2.00000000 sec
D11 0.03000000 sec
TD0 1
SFO1 75.5054723 MHz
NUC1 13C
P1 10.00 usec
PLW1 47.66699982 W
SFO2 300.2512010 MHz
NUC2 1H
CPDPRG[2 waltz16
PCPD2 90.00 usec
PLW2 7.94199991 W
PLW12 0.19330969 W
PLW13 0.09688683 W
F2 - Processing parameters
SI 32768
SF 75.4979225 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.40
HN
O
O
O
NH2
N
N
NH
O
N
HN
O
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 ppm

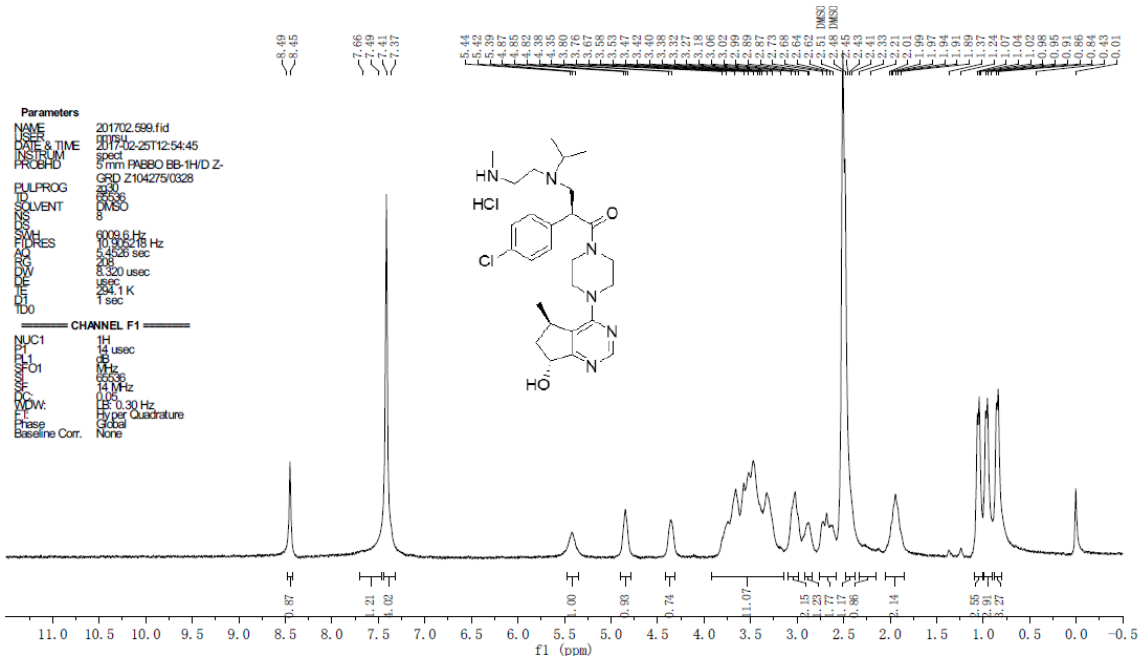
Compound **12:**

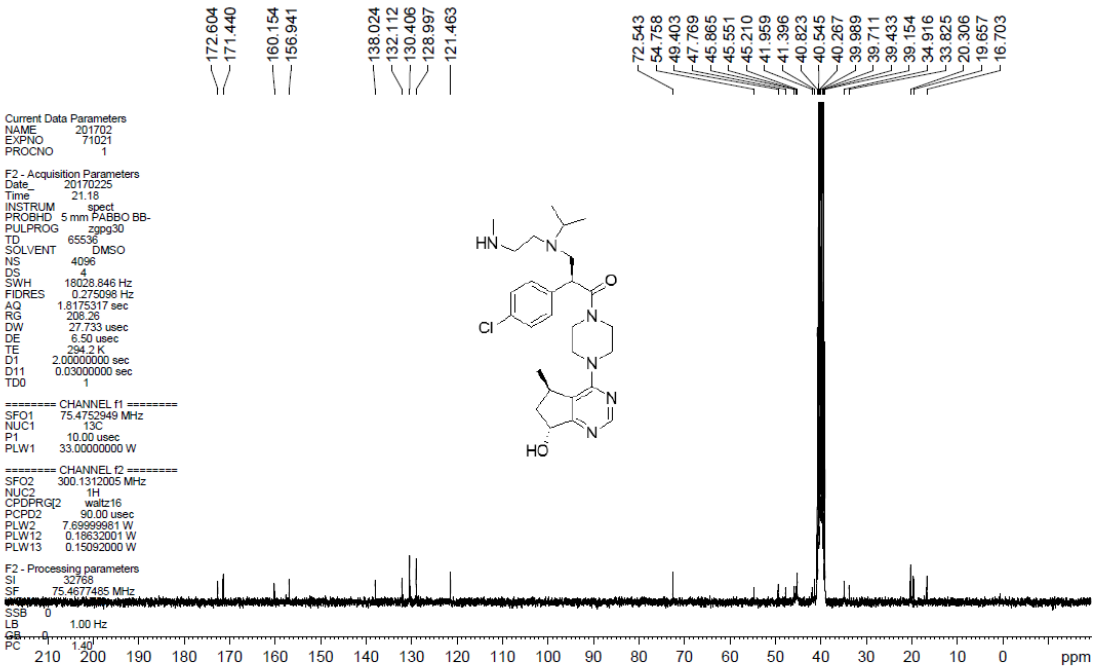

8.602
8.556
7.700
7.670
7.496
7.472
7.411
7.384
7.356
7.329
7.296
7.287
7.248
7.229
7.202
7.035
7.007
6.880
6.855
5.033
4.993
4.870
4.276
4.236
4.169
4.133
3.977
3.803
3.785
3.743
3.727
3.628
3.596
3.578
3.458
3.445
3.428
3.386
3.368
3.350
3.306
3.085
3.046
2.999
2.981
2.831
2.802
2.789
2.539
2.496
2.423
2.280
2.235
1.787
1.750
1.387
1.376
1.353
0.198
-0.000
Current Data Parameters
NAME 201703
EXPNO 610
PROCNO 1
F2 - Acquisition Parameters
Date_ 20170324
Time 13.57
INSTRUM spect
PROBHD 5 mm PABBO BB-
PULPROG zg30
TD 65536
SOLVENT CD3OD
NS 8
DS 0
SWH 6009.615 Hz
FIDRES 0.091699 Hz
AQ 5.4525952 sec
RG 208.26
DW 83.200 usec
DE 6.50 usec
TE 294.8 K
D1 1.00000000 sec
TD0 1
======== CHANNEL f1 ========
SFO1 300.1318534 MHz
NUC1 1H
P1 14.00 usec
PLW1 7.69999981 W
F2 - Processing parameters
SI 65536
SF 300.1300071 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00
HN
O
O
O
N
H
O
N
N
NH
O
N
HN
O
11 10 9 8 7 6 5 4 3 2 1 0 ppm
0.899
0.972
1.129
11.097
1.099
1.026
1.106
2.090
2.342
2.416
7.043
3.082
2.145
2.153
2.078
2.098
0.346

Compound **13:**

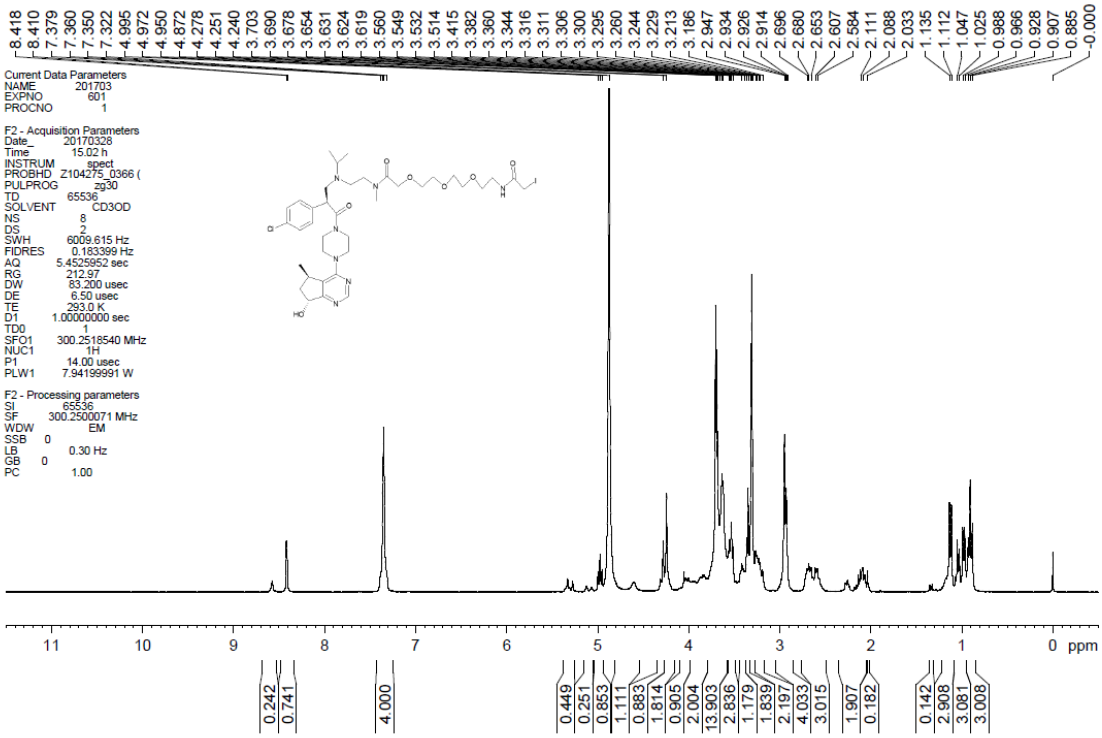


Compound **14:**




172.604
171.440
160.154
156.941
138.024
132.112
130.406
128.997
121.463
72.543
54.758
49.403
47.769
45.865
45.551
45.210
41.959
41.396
40.823
40.545
40.267
39.989
39.711
39.433
39.154
34.916
33.825
20.306
19.657
16.703
Current Data Parameters
NAME 201702
EXPNO 71021
PROCNO 1
F2 - Acquisition Parameters
Date_ 20170225
Time 21.18
INSTRUM spect
PROBHD 5 mm PABBO BB-
PULPROG zgpg30
TD 65536
SOLVENT DMSO
NS 4096
DS 4
SWH 18028.846 Hz
FIDRES 0.275098 Hz
AQ 1.8175317 sec
RG 208.26
DW 27.733 usec
DE 6.50 usec
TE 294.2 K
D1 2.00000000 sec
D11 0.03000000 sec
TD0 1
======== CHANNEL f1 ========
SFO1 75.4752949 MHz
NUC1 13C
P1 10.00 usec
PLW1 33.00000000 W
======== CHANNEL f2 ========
SFO2 300.1312005 MHz
NUC2 1H
CPDPRG[2 waltz16
PCPD2 90.00 usec
PLW2 7.69999981 W
PLW12 0.18632001 W
PLW13 0.15092000 W
F2 - Processing parameters
SI 32768
SF 75.4677485 MHz
SSB 0
LB 1.00 Hz
GB 0
PC 1.40
HN
N
O
Cl
N
N
N
HO
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 ppm

Compound **16:**


8.418
8.410
7.379
7.360
7.350
7.322
4.995
4.972
4.950
4.872
4.278
4.251
4.240
3.703
3.690
3.678
3.654
3.631
3.624
3.619
3.560
3.549
3.532
3.514
3.415
3.382
3.360
3.344
3.316
3.311
3.306
3.300
3.295
3.260
3.244
3.229
3.213
3.186
2.947
2.934
2.926
2.914
2.696
2.680
2.653
2.607
2.584
2.111
2.088
2.033
1.135
1.112
1.047
1.025
0.988
0.966
0.928
0.907
0.885
-0.000
Current Data Parameters
NAME 201703
EXPNO 601
PROCNO 1
F2 - Acquisition Parameters
Date_ 20170328
Time 15.02 h
INSTRUM spect
PROBHD Z104275_0366 (
PULPROG zg30
TD 65536
SOLVENT CD3OD
NS 8
DS 2
SWH 6009.615 Hz
FIDRES 0.183399 Hz
AQ 5.4525952 sec
RG 212.97
DW 83.200 usec
DE 6.50 usec
TE 293.0 K
D1 1.00000000 sec
TD0 1
SFO1 300.2518540 MHz
NUC1 1H
P1 14.00 usec
PLW1 7.94199991 W
F2 - Processing parameters
SI 65536
SF 300.2500071 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00
11 10 9 8 7 6 5 4 3 2 1 0 ppm
0.242
0.741
4.000
0.449
0.251
0.853
1.111
0.883
1.814
0.905
2.004
13.903
2.836
1.179
1.839
2.197
4.033
3.015
1.907
0.182
0.142
2.908
3.081
3.008

[1] Weisner, J.; Gontla, R.; van der Westhuizen, L.; Oeck, S.; Ketzer, J.; Janning, P.; Richters, A.; Mühlenberg, T.; Fang, Z.; Taher, A.; Jendrossek, V.; Pelly, S. C.; Bauer, S.; van Otterlo, W. A. L.; Rauh, D. "Covalent-Allosteric Kinase Inhibitors" *Angew. Chem. Int. Ed.* **2015**, *54*, 10313-10316.

[2] Bilodeau, M. T.; Balitza, A. E.; Hoffman, J. M.; Manley, P. J.; Barnett, S. F.; Defeo-Jones, D.; Haskell, K.; Jones, R. E.; Leander, K.; Robinson, R. G.; Smith, A. M.; Huber, H. E.; Hartman, G. D. "Allosteric inhibitors of Akt1 and Akt2: a naphthyridinone with efficacy in an A2780 tumor xenograft model" *Bioorg. Med. Chem. Lett.* **2008**, *18*, 3178-3182. (c) Blake, J. F.; Xu, R.; Bencsik, J. R.; Xiao, D.; Kallan, N. C.; Schlachter, S.; Mitchell, I. S.; Spencer, K. L.; Banka, A. L.; Wallace, E. M.; Gloor, S. L.; Martinson, M.; Woessner, R. D.; Vigers, G. P. A.; Brandhuber, B. J.; Liang, J.; Safina, B. S.; Li, J.; Zhang, B.; Chabot, C.; Do, S.; Lee, L.; Oeh, J.; Sampath, D.; Lee, B. B.; Lin, K.; Liederer, B. M.; Skelton, N. J. "Discovery and preclinical pharmacology of a selective ATP-competitive Akt inhibitor (GDC-0068) for the treatment of human tumors" *J. Med. Chem.* **2012**, *55*, 8110-8127

[3] Buckley, D., L.; Raina, K.; Darricarrer, N.; Hines, J.; Gustafson, J. J.; Smith, I. E.; Miah, A. H.; Harling, J. D.; Crews, C. M. "HaloPROTACS: use of small molecule PROTACs to induce degradation of HaloTag fusion proteins" *ACS Chem. Bio.* **2015**, *10*, 1831-1837.