

PROTACs Suppression Of CDK4/6, Crucial Kinases For Cell Cycle Regulation In Cancer

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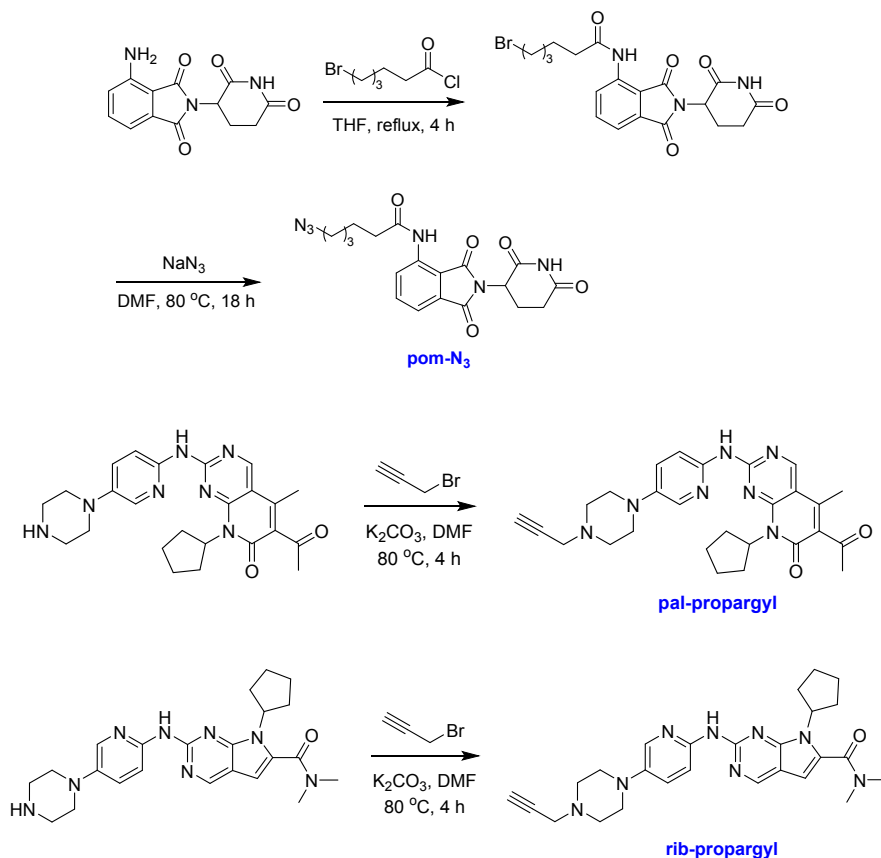
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A. General Experimental Information

All reactions were carried out under an inert atmosphere (nitrogen or argon where stated) with dry solvents under anhydrous conditions. Glassware for anhydrous reactions was dried in an oven at 140 °C for minimum 6 h prior to use. Dry solvents were obtained by passing the previously degassed solvents through activated alumina columns. Yields refer to chromatographically and spectroscopically (¹H-NMR) homogeneous materials, unless otherwise stated. Reagents were purchased at a high commercial quality (typically 97 % or higher) and used without further purification, unless otherwise stated. Analytical thin layer chromatography (TLC) was carried out on Merck silica gel plates with QF-254 indicator and visualized by UV. Flash column chromatography was performed using silica gel 60 (Silicycle, 230-400 mesh). ¹H and ¹³C spectra were recorded on a 400 MHz spectrometer and were calibrated using residual non-deuterated solvent as an internal reference. The following abbreviations or combinations thereof were used to explain the multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublet, ddd = doublet of doublet of doublets.

Palbociclib and Ribociclib were purchased from LC Laboratories. MDA-MB-231, MCF-7 and U87-MG cells (from American Type Culture Collection) were cultured on 75 cm² culture flasks in Dulbecco's Modified Eagle Medium/nutrient mixture F-12 (DMEM/F12, Sigma Chemical, St. Louis, MO) supplemented with 10 % FBS. CDK4, CDK6 and Phospho-Rb (Ser780) mAb were purchased from Cell Signaling Technology. Goat anti-rabbit (H+L) secondary antibody (HRP conjugated) were purchased from ThermoFisher Scientific. SuperSignal West Dura Substrate (ThermoFisher Scientific) was used as Western blot substrate.

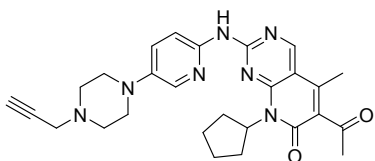
B. Synthesis of Intermediates and PROTACs



Scheme S1. Syntheses of **pom-N₃**, **pal-propargyl** and **rib-propargyl**.

Pom-N₃ was made according to literature^[1].

Synthesis of 6-acetyl-8-cyclopentyl-5-methyl-2-((5-(4-(prop-2-yn-1-yl)piperazin-1-yl)pyridin-2-yl)amino)pyrido[2,3-d]pyrimidin-7(8H)-one (**pal-propargyl**)



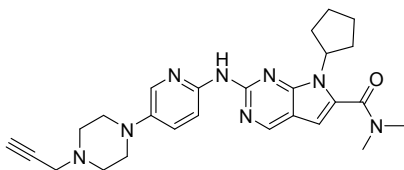
To a solution of Palbociclib (0.25 mmol) and K₂CO₃ (0.50 mmol) in 2 mL DMF was added propargyl bromide (0.30 mmol). The mixture was heated to 80 °C and stirred for 4 h. Solvent was removed. H₂O was added to the mixture and the crude product was purified by prep-HPLC to obtain **pal-propargyl** as a yellow solid (110 mg, 90 %).

^1H NMR (400 MHz, MeOD) δ 9.08 (d, J = 4.2 Hz, 1H), 8.15 – 7.99 (m, 2H), 7.81 – 7.67 (m, 1H), 6.06 – 5.97 (m, 1H), 4.69 (dd, J = 10.6, 2.4 Hz, 1H), 4.15 (d, J = 2.5 Hz, 1H), 4.07 – 3.85 (m, 2H), 3.72 (dd, J = 12.3, 7.2 Hz, 1H), 3.64 – 3.49 (m, 6H), 3.38 (t, J = 2.5 Hz, 1H), 2.52 (s, 3H), 2.44 (d, J = 1.3 Hz, 3H), 2.33 (dt, J = 15.2, 7.8 Hz, 2H), 2.10 (t, J = 6.7 Hz, 2H), 1.96 – 1.88 (m, 2H), 1.72 (dd, J = 10.5, 5.5 Hz, 2H).

^{13}C NMR (101 MHz, MeOD) δ 202.6, 161.2, 157.3, 155.8, 144.3, 142.1, 141.7, 132.2, 116.0, 115.8, 109.7, 92.3, 82.9, 79.2, 72.3, 54.1, 50.7, 46.2, 45.3, 30.0, 27.6, 25.3, 12.7.

HRMS (ESI+) m/z calcd for $\text{C}_{27}\text{H}_{32}\text{N}_7\text{O}_2^+$ ($\text{M}+\text{H}$) $^+$ 486.2612; found 486.2604.

Synthesis of 7-cyclopentyl-N,N-dimethyl-2-((5-(4-(prop-2-yn-1-yl)piperazin-1-yl)pyridin-2-yl)amino)-7H-pyrrolo[2,3-d]pyrimidine-6-carboxamide (**rib-propargyl**)



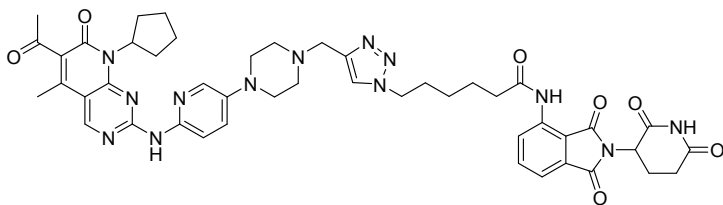
Rib-propargyl was synthesized in a similar way as **pal-propargyl** using Ribociclib instead of Palbociclib. Light yellow solid (105 mg, 89 %).

^1H NMR (400 MHz, MeOD) δ 8.99 (d, J = 1.3 Hz, 1H), 8.11 – 7.96 (m, 2H), 7.47 (d, J = 9.4 Hz, 1H), 6.84 (s, 1H), 6.07 (d, J = 6.3 Hz, 1H), 4.69 (dd, J = 10.0, 2.4 Hz, 1H), 4.19 (d, J = 2.5 Hz, 1H), 3.95 (ddd, J = 45.4, 37.3, 13.2 Hz, 2H), 3.70 (dt, J = 4.9, 3.9 Hz, 1H), 3.64 – 3.45 (m, 6H), 3.40 (t, J = 2.5 Hz, 1H), 3.18 (d, J = 8.3 Hz, 6H), 2.48 (dd, J = 12.3, 8.4 Hz, 2H), 2.20 – 2.05 (m, 4H), 1.76 (d, J = 5.6 Hz, 2H).

^{13}C NMR (101 MHz, MeOD) δ 163.4, 152.4, 151.2, 145.9, 145.5, 141.3, 136.6, 134.1, 125.0, 115.1, 114.9, 101.1, 79.7, 71.8, 58.3, 50.7, 46.2, 38.2, 34.0, 30.1, 24.2.

HRMS (ESI+) m/z calcd for $\text{C}_{26}\text{H}_{33}\text{N}_8\text{O}^+$ ($\text{M}+\text{H}$) $^+$ 473.2772; found 473.2775.

Synthesis of 6-(4-((4-(6-((6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d]pyrimidin-2-yl)amino)pyridin-3-yl)piperazin-1-yl)methyl)-1H-1,2,3-triazol-1-yl)-N-(2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)hexanamide (**pal-pom**)



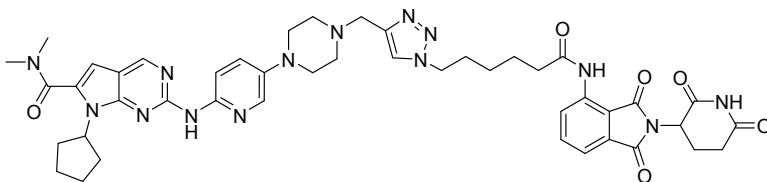
CuSO₄ (6 μmol), Na ascorbate (24 μmol) and TBTA (6 μmol) were added to a mixture of **pal-propargyl** (20 μmol) and **pom-N₃** (22 μmol) in 0.4 mL DMSO and 0.2 mL H₂O. The reaction mixture was stirred overnight at room temperature. Solvent was removed. H₂O was added to the mixture and the crude product was purified by prep-HPLC to obtain **pal-pom** as a yellow solid (8.6 mg, 48 %).

¹H NMR (400 MHz, MeOD) δ 9.04 (s, 1H), 8.59 (d, *J* = 8.4 Hz, 1H), 8.21 (s, 1H), 7.98 (d, *J* = 2.3 Hz, 1H), 7.92 (d, *J* = 9.3 Hz, 1H), 7.78 (dd, *J* = 12.6, 5.1 Hz, 2H), 7.57 (d, *J* = 7.3 Hz, 1H), 6.00 (p, *J* = 8.8 Hz, 1H), 5.15 (dd, *J* = 12.4, 5.5 Hz, 1H), 4.58 – 4.49 (m, 4H), 3.49 (s, 7H), 2.95 – 2.67 (m, 4H), 2.56 – 2.50 (m, 5H), 2.43 (s, 3H), 2.33 (dt, *J* = 15.7, 7.9 Hz, 3H), 2.23 – 2.01 (m, 6H), 1.97 – 1.66 (m, 7H), 1.45 (dq, *J* = 15.3, 7.7 Hz, 2H).

¹³C NMR (101 MHz, MeOD) δ 202.5, 173.0, 172.8, 170.1, 168.4, 166.8, 161.2, 156.9, 155.8, 155.5, 144.1, 141.8, 141.6, 136.7, 136.0, 135.7, 134.3, 132.6, 131.6, 126.6, 125.6, 124.5, 118.1, 116.9, 116.2, 110.0, 54.2, 50.8, 50.3, 50.0, 49.2, 45.7, 36.4, 30.7, 30.0, 29.4, 27.6, 25.5, 25.3, 24.1, 22.2, 12.8.

HRMS (MALDI+) *m/z* calcd for C₄₆H₅₂N₁₃O₇⁺ (M+H)⁺ 898.4107; found 898.4092.

Synthesis of 7-cyclopentyl-2-((5-(4-((1-(6-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)-6-oxohexyl)-1H-1,2,3-triazol-4-yl)methyl)piperazin-1-yl)pyridin-2-yl)amino)-N,N-dimethyl-7H-pyrrolo[2,3-d]pyrimidine-6-carboxamide (**rib-pom**)



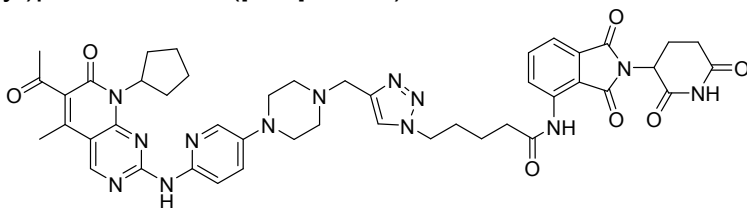
Rib-pom was synthesized in a similar way as **pal-pom** using **pal-propargyl** instead of **rib-propargyl**. Light yellow solid (6.7 mg, 38 %).

^1H NMR (400 MHz, MeOD) δ 8.96 (s, 1H), 8.57 (d, J = 8.4 Hz, 1H), 8.24 (s, 1H), 7.99 (dd, J = 9.5, 2.9 Hz, 1H), 7.93 (d, J = 2.8 Hz, 1H), 7.76 (dd, J = 8.4, 7.4 Hz, 1H), 7.54 (d, J = 7.2 Hz, 1H), 7.42 (d, J = 9.4 Hz, 1H), 6.83 (s, 1H), 5.15 (dd, J = 12.6, 5.5 Hz, 1H), 4.58 (s, 2H), 4.52 (t, J = 6.9 Hz, 2H), 3.54 (s, 8H), 3.18 (d, J = 7.8 Hz, 6H), 2.99 – 2.65 (m, 4H), 2.49 (dt, J = 12.4, 7.8 Hz, 4H), 2.22 – 1.93 (m, 9H), 1.77 (dt, J = 15.0, 7.4 Hz, 4H), 1.45 (dq, J = 15.5, 7.8 Hz, 2H).

^{13}C NMR (101 MHz, MeOD) δ 173.1, 172.8, 170.1, 168.4, 166.9, 163.4, 152.4, 151.1, 145.8, 145.4, 141.2, 136.7, 136.6, 135.9, 135.7, 134.0, 131.6, 126.6, 125.6, 125.0, 118.1, 116.9, 115.1, 114.9, 101.1, 58.3, 50.8, 50.3, 50.0, 49.2, 46.0, 36.4, 34.0, 30.7, 30.1, 29.4, 25.5, 24.2, 24.1, 22.2.

HRMS (MALDI+) m/z calcd for $\text{C}_{45}\text{H}_{53}\text{N}_{14}\text{O}_6^+$ ($\text{M}+\text{H}$) $^+$ 885.4267; found 885.4245.

Synthesis of 5-(4-((4-(6-((6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d]pyrimidin-2-yl)amino)pyridin-3-yl)piperazin-1-yl)methyl)-1H-1,2,3-triazol-1-yl)-N-(2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)pentanamide (**pal-pom-2**)

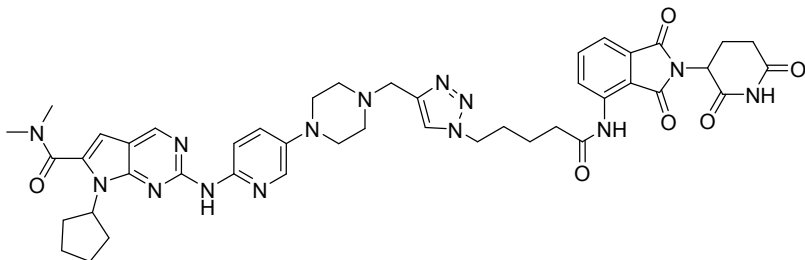


Pal-pom-2 was synthesized in a similar way as **pal-pom** using **pom-N₃** analog with one less -CH₂. Yellow solid (8.8 mg, 52 %).

¹H NMR (400 MHz, MeOD) δ 9.07 (s, 1H), 8.56 (d, *J* = 8.0 Hz, 1H), 8.27 (s, 1H), 8.07 – 7.95 (m, 2H), 7.77 (dd, *J* = 8.4, 7.4 Hz, 1H), 7.68 (d, *J* = 9.3 Hz, 1H), 7.58 (d, *J* = 6.8 Hz, 1H), 6.07 – 5.95 (m, 1H), 5.15 (dd, *J* = 12.5, 5.5 Hz, 1H), 4.62 – 4.51 (m, 4H), 3.56 (s, 7H), 2.96 – 2.66 (m, 3H), 2.57 (t, *J* = 7.2 Hz, 2H), 2.51 (d, *J* = 3.1 Hz, 3H), 2.45 – 2.27 (m, 6H), 2.22 – 2.00 (m, 7H), 1.97 – 1.62 (m, 7H).

HRMS (MALDI+) *m/z* calcd for C₄₅H₅₀N₁₃O₇⁺ (*M*+H)⁺ 884.3951; found 884.3928.

Synthesis of 7-cyclopentyl-2-((5-(4-((1-(5-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)-5-oxopentyl)-1H-1,2,3-triazol-4-yl)methyl)piperazin-1-yl)pyridin-2-yl)amino)-N,N-dimethyl-7H-pyrrolo[2,3-d]pyrimidine-6-carboxamide (**rib-pom-2**)



Rib-pom-2 was synthesized in a similar way as **rib-pom** using **pom-N₃** analog with one less -CH₂. Light yellow solid (6.9 mg, 42 %).

¹H NMR (400 MHz, MeOD) δ 8.97 (s, 1H), 8.56 (d, *J* = 8.4 Hz, 1H), 8.28 (s, 1H), 8.05 – 7.92 (m, 2H), 7.82 – 7.73 (m, 1H), 7.56 (d, *J* = 7.3 Hz, 1H), 7.42 (d, *J* = 9.4 Hz, 1H), 6.84 (s, 1H), 5.15 (dd, *J* = 12.6, 5.5 Hz, 1H), 4.58 (dd, *J* = 13.6, 6.8 Hz,

C. Biological Assays

Kinase Binding Affinity Assay^[2]

Kinase-tagged T7 phage strains were prepared in an *E. coli* host derived from the BL21 strain. *E. coli* were grown to log-phase and infected with T7 phage and incubated with shaking at 32°C until lysis. The lysates were centrifuged and filtered to remove cell debris. The remaining kinases were produced in HEK-293 cells and subsequently tagged with DNA for qPCR detection. Streptavidin-coated magnetic beads were treated with biotinylated small molecule ligands for 30 minutes at room temperature to generate affinity resins for kinase assays. The liganded beads were blocked with excess biotin and washed with blocking buffer (SeaBlock (Pierce), 1% BSA, 0.05% Tween 20, 1 mM DTT) to remove unbound ligand and to reduce nonspecific binding. Binding reactions were assembled by combining kinases, liganded affinity beads, and test compounds in 1x binding buffer (20% SeaBlock, 0.17x PBS, 0.05% Tween 20, 6 mM DTT). Test compounds were prepared as 111X stocks in 100% DMSO. K_d s were determined using an 11-point 3-fold compound dilution series with three DMSO control points. All compounds for K_d measurements are distributed by acoustic transfer (non-contact dispensing) in 100% DMSO. The compounds were then diluted directly into the assays such that the final concentration of DMSO was 0.9%. All reactions performed in polypropylene 384-well plate. Each was a final volume of 0.02 ml. The assay plates were incubated at room temperature with shaking for 1 hour and the affinity beads were washed with wash buffer (1x PBS, 0.05% Tween 20). The beads were then re-suspended in elution buffer (1x PBS, 0.05% Tween 20, 0.5 μ M non-biotinylated affinity ligand) and incubated at room temperature with shaking for 30 minutes. The kinase concentration in the eluates was measured by qPCR.

Protein Degradation (Western Blot)

Cells were seeded in 24-well plate (80,000 cells/well) and allowed to adhere overnight. Culturing media were replaced by fresh media with PROTACs (DMSO < 0.5%). Cells were incubated for certain time (varies according to different experiments, see below) before lysed by RIPA buffer (Pierce) according to manufacturer's instructions. Total protein concentrations were determined and calibrated by BCA protein assay (Pierce). Whole cell lysates were subjected to SDS-PAGE, transferred to PVDF membrane and proceeded to Western blot protocol: membrane was blocked with SuperBlock (TBS) Blocking Buffer (ThermoFisher Scientific) for 1 h at room temperature, incubated with primary antibodies overnight at 4 °C, washed with TBS-T (TBS + 0.05% Tween 20) 3 times, incubated with secondary antibodies for 1 h at room temperature, washed with TBS-T 4-6 times. Afterwards, blots were treated with SuperSignal West Dura Substrate (ThermoFisher Scientific) and imaged by ChemiDoc XRS (BioRad) imaging system.

For time course study, MDA-MB-231 cells were treated with PROTACs or 0.3% DMSO for different time period before cell lysis. For washout experiment, cells were treated with PROTACs or 0.3% DMSO for 18 h and washed with warm PBS twice to remove PROTACs residue. Afterwards, cells were incubated for different time period before cell lysis. For rescue experiment, cells were pretreated with palbociclib, pomalidomide, MLN4924, MG-132 or 0.1% DMSO for 2 h before treated with PROTACs or 0.3% DMSO for 18 h and cell lysis.

Dilution factor: anti-CDK4 mAb (1:1000), anti-CDK6 mAb (1:1000), anti-phospho-Rb (Ser 780) mAb (1:1000), anti-b-actin mAb (1:5000), HRP-conjugated anti-rabbit IgG (H+L) (1:50,000).

Cytotoxicity Assay

MDA-MB-231 or MCF-7 cells were seeded in 96-well plate (5,000 cells/well) in 50 μ L DMEM/F12 media with 10% FBS and allowed to adhere overnight. Compounds were diluted in PFHM-II media and added to each well. Cells were incubated for 72 h before AlamarBlue reagent (Invitrogen) was added. Cells were incubated for an additional 2 h and fluorescence intensity (Ex/Em 560/590 nm) was measured by a BioTek Synergy 4 Microplate Reader. Cell viabilities are calculated as: % live cells = $OD_{\text{compound}}/OD_{\text{DMSO}} \times 100$. Results are processed by GraphPad Prism 6.0 software.

Cell proliferation Assay

MCF-7 cells were seeded in 24-well plate (20,000 cells/well) in 0.5 mL DMEM/F12 media with 10% FBS and allowed to adhere overnight. Compounds were diluted in the same media and added to each well. Media were refreshed every 2 days with the exact same components in each well. At day 2, 4 and 6, AlamarBlue reagent (Invitrogen) was added. Cells were incubated for an additional 2 h and fluorescence intensity (Ex/Em 560/590 nm) was measured by a BioTek Synergy 4 Microplate Reader. Percentage cell proliferation was calculated as $OD_{\text{compound}}/OD_{\text{DMSO at day 2}} \times 100$. Results are processed by GraphPad Prism 6.0 software.

To clarify, the cytotoxicity assay (Figure S4) was performed using a shorter incubation time than the proliferation assay, and with a range of drug concentrations to get a S-curve. In the proliferation assay (Figure S6), the drug dose was set at 3 μ M (not cytotoxic, Figure S4b) but the incubation was carried on for a longer time to monitor the long-term effects.

D. Results and discussion

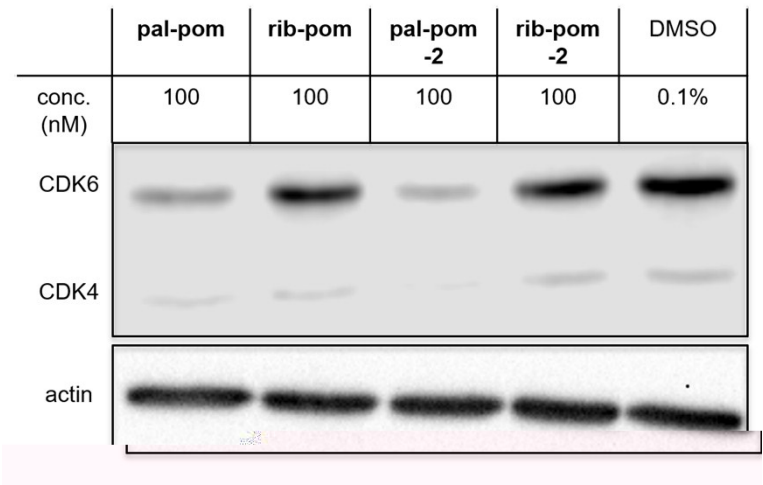


Figure S1. CDK4/6 degradation using different linkers on MDA-MB-231 cells. Similar results were observed when different length of linkers were applied.

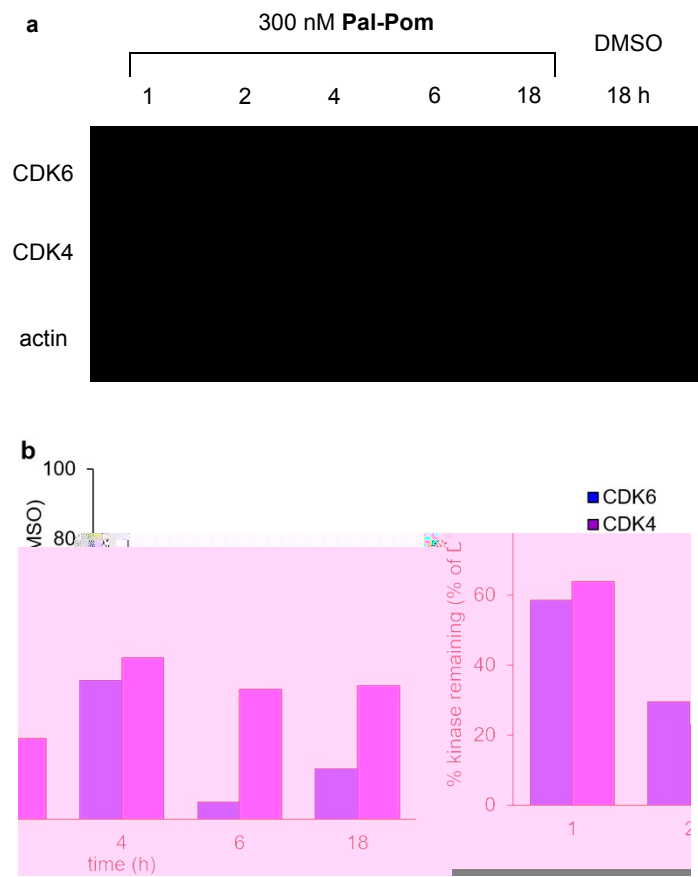


Figure S2. **a** Kinetics for depletion of CDK4/6 on MDA-MB-231 cells with 300 nM **pal-pom**. **b** Quantified data for **a** (normalized to DMSO as 100%).

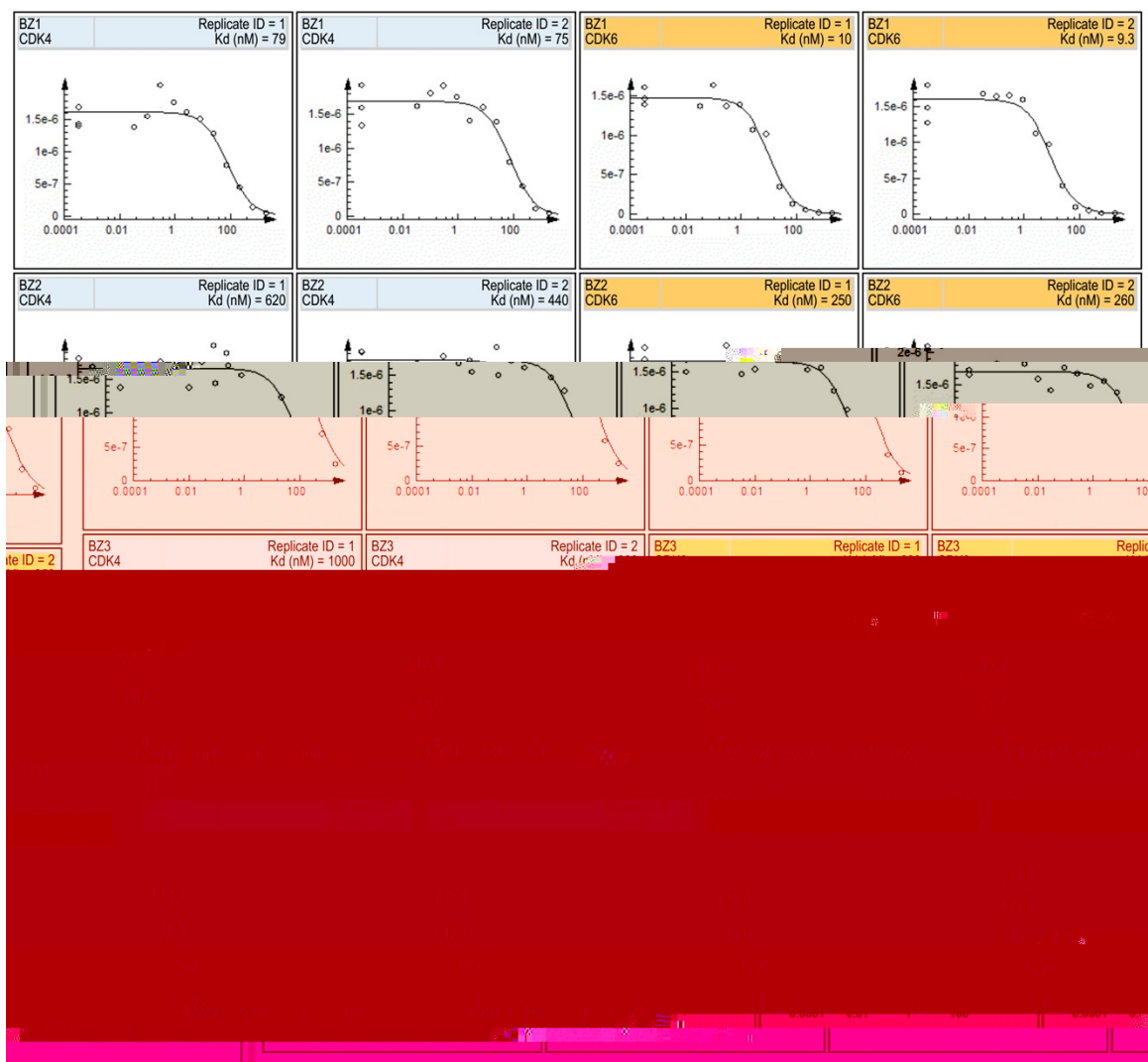


Figure S3. Kinase Binding Affinity Assay on CDK4/6, each K_d was determined in duplicate experiments. BZ1 = **palbociclib**, BZ2 = **ribociclib**, BZ3 = **pal-pom**, BZ4 = **rib-pom**.

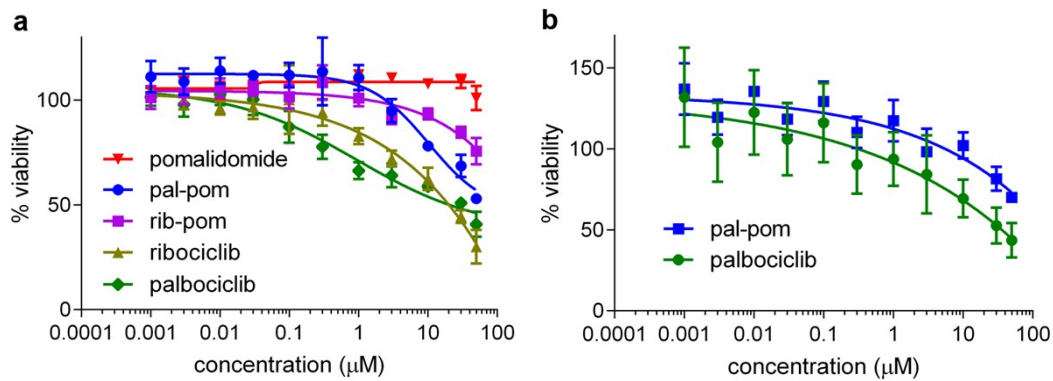


Figure S4. Cytotoxicity assay on MDA-MB-231 cells (a) and MCF-7 cells (b) (72 h incubation).

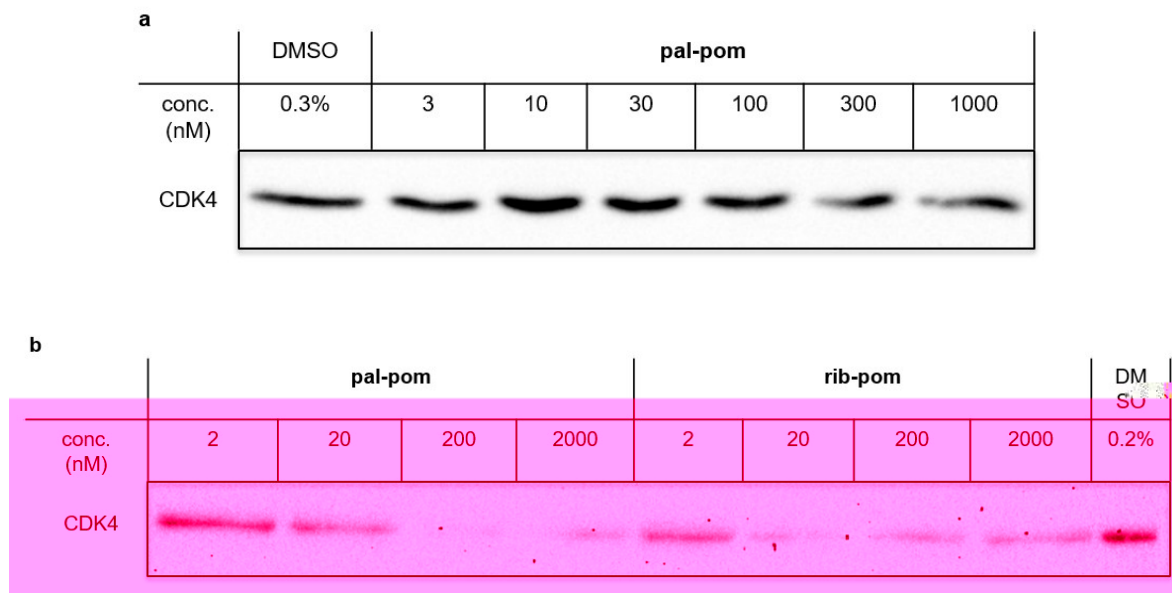


Figure S5. CDK4 depletion (Western blot) on MCF-7 cells (a) and U87 cells (b) with 18 h incubation of PROTACs. (CDK6 was not detected in these two cell lines by the same mAb used in MDA-MB-231 cell line)

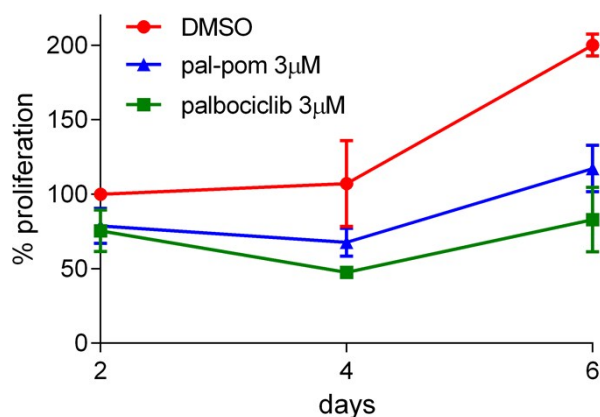


Figure S6. Cell proliferation assay on MCF-7 cell line. Culturing media were refreshed (with DMSO, **pal-pom** or palbociclib) every 2 days. Reading of DMSO control set at day 2 was used as 100% standard.

Table S1. Summary of some reported PROTACs and their estimated DC₅₀ value based on Western blots.

Protein	CDK8 ^[3]	CDK9 ^[4]	HDAC6 ^[5]	BRD4 ^[6]	BRD3 ^[6]	BRD2 ^[6]	ALK ^[7]	PI3K ^[1]	Sirt2 ^[8]	BTK ^[9]
DC ₅₀ ^[a]	significant at 1 μM	~ 10 μM	100-300 nM	0.1-0.3 nM	30-100 nM	100-300 nM	30-60 nM	20-50 μM	0.2-1 μM	25-100 nM

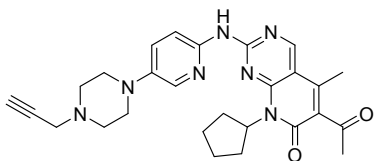
[a] Estimated based on Western blots in corresponding papers.

E. References

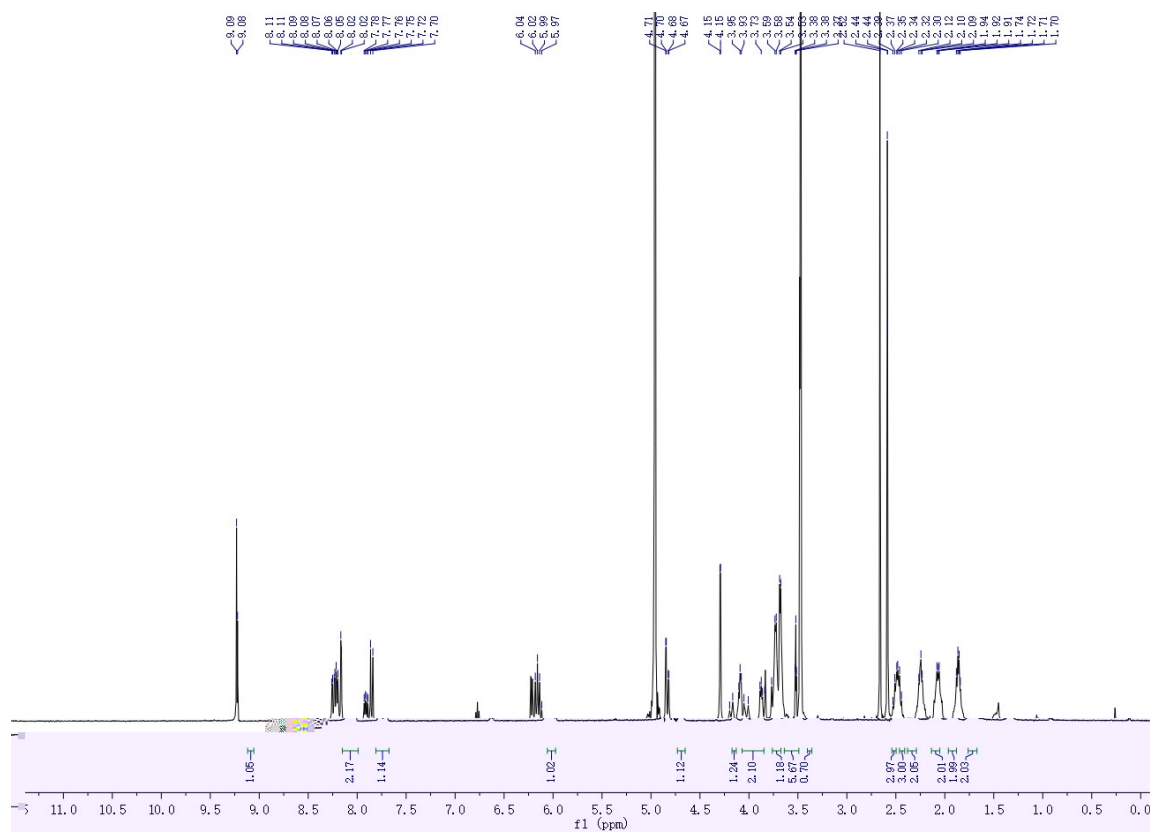
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- [2] Adopted from DiscoverX company KINOMEScan™ protocol.
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F. NMRs

Pal-propargyl



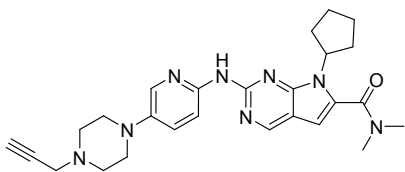
^1H NMR in MeOD



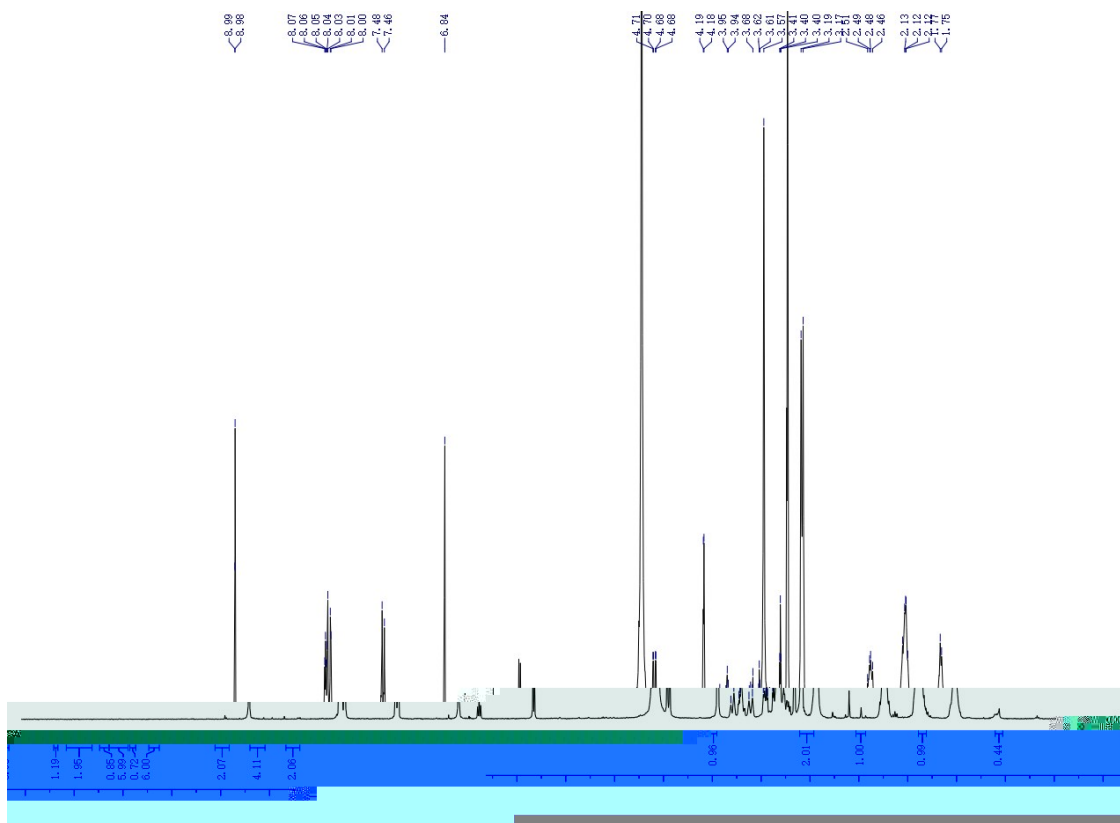
^{13}C NMR in MeOD



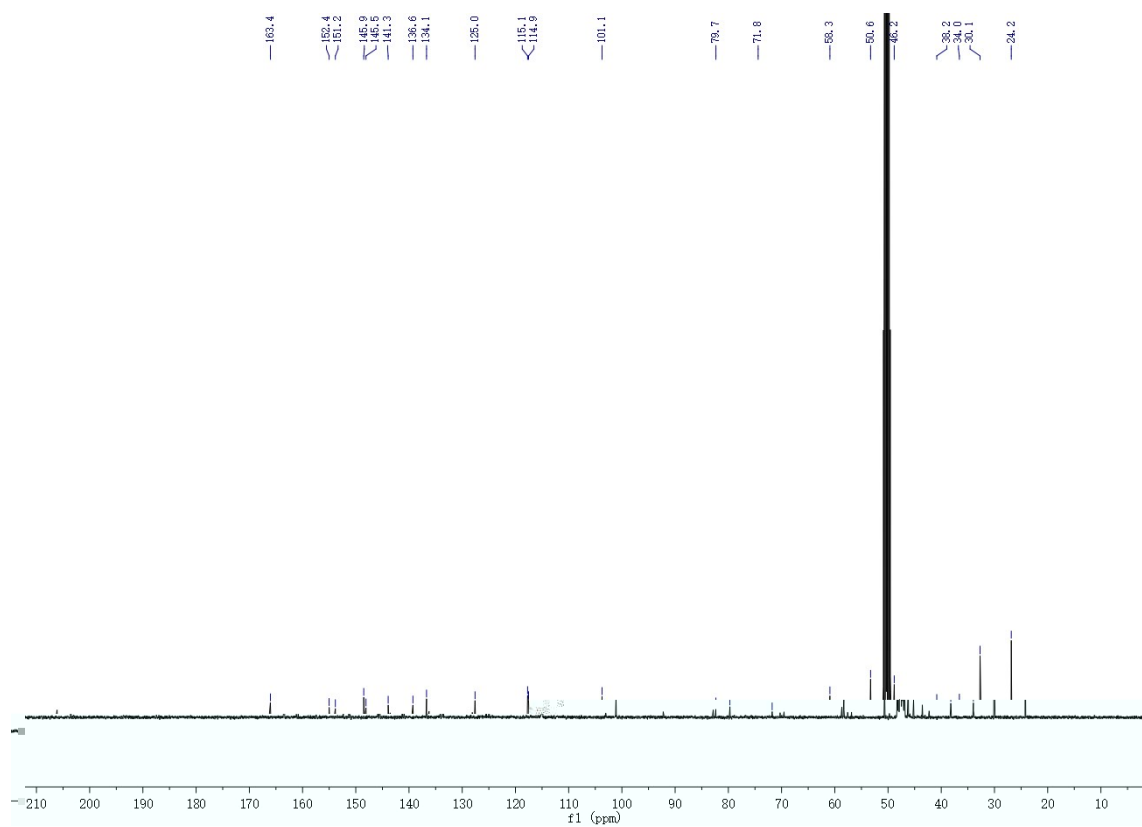
Rib-propargyl



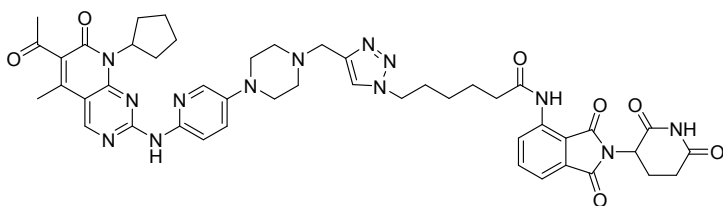
^1H NMR in MeOD



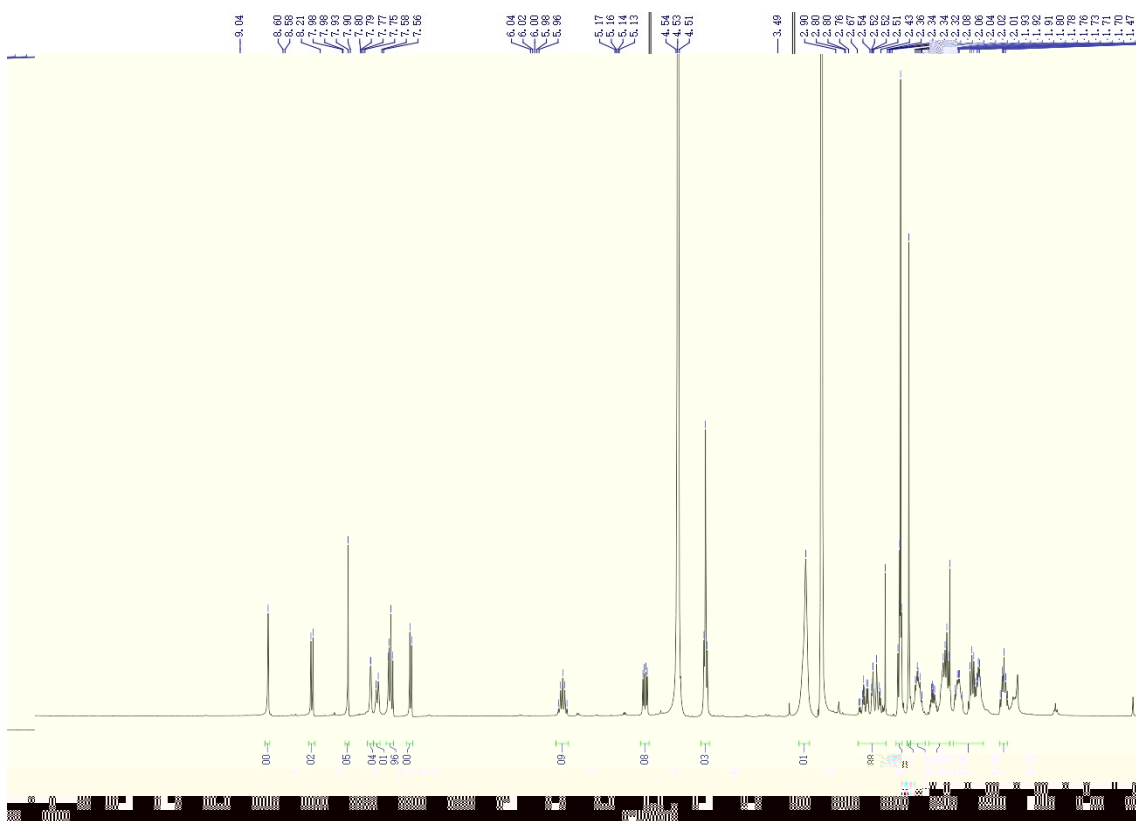
^{13}C NMR in MeOD



Pal-pom



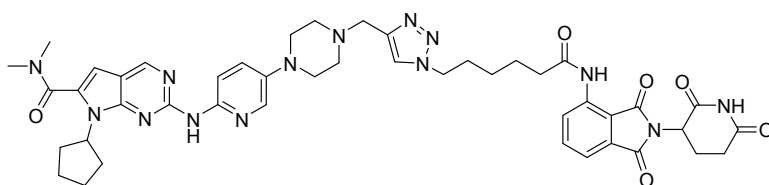
^1H NMR in MeOD



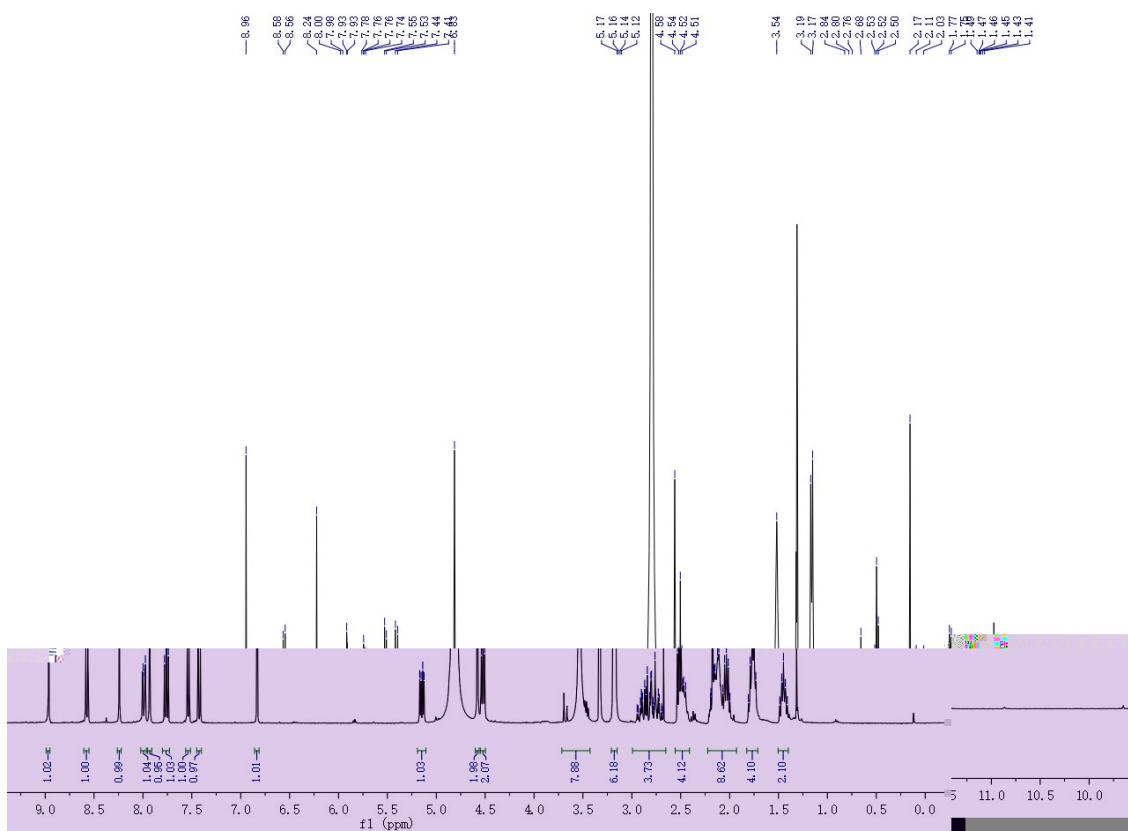
^{13}C NMR in MeOD



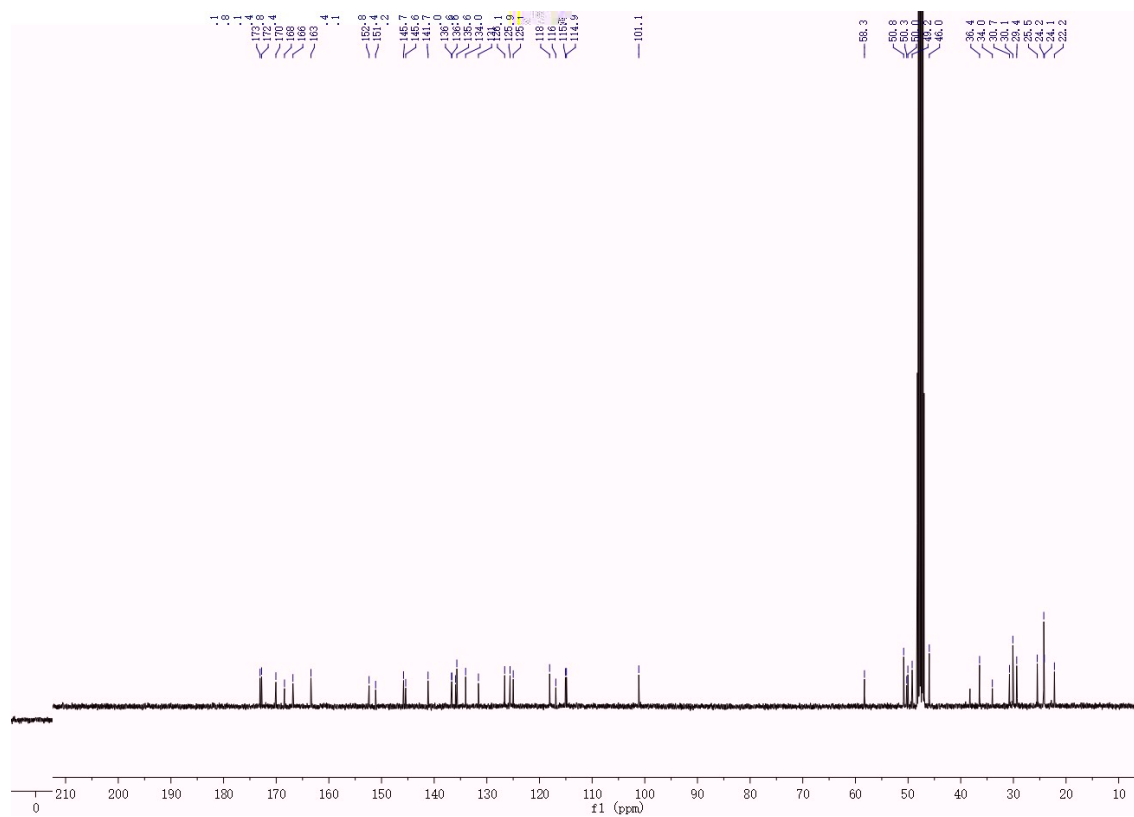
Rib-pom



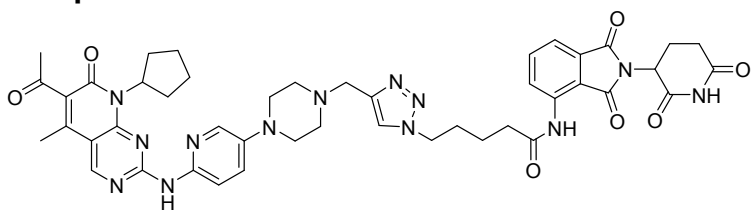
^1H NMR in MeOD



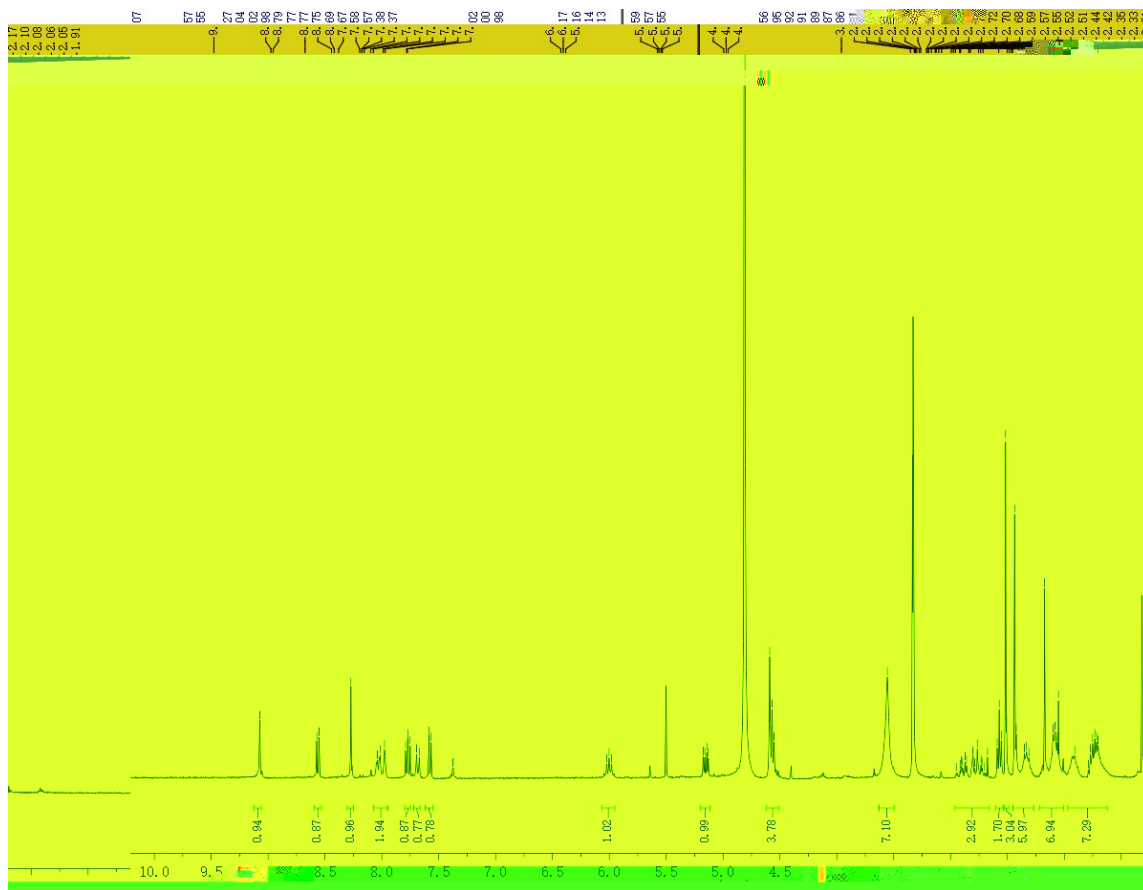
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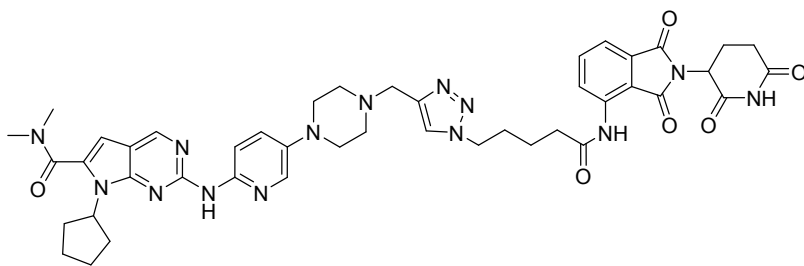
Pal-pom-2



^1H NMR in MeOD



Rib-pom-2



^1H NMR in MeOD

