Supporting Information

Facile Synthesis of Rapamycin-Peptide Conjugates as mTOR and Akt inhibitor.

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1) General information:

The Fmoc protected amino acids and Wang resin (100-200 mesh) used in solid phase peptide synthesis were purchased from Novabiochem. Rapamycin was purchased from APExBIO. The flame-dried or oven dried glassware were used to carried out the reactions. All the reactions were performed with magnetic stirring under nitrogen atmosphere using freshly dry and distilled solvents, unless otherwise noted. Readymade TLC silica gel 60 F_{254} plates (Merck, Dermstadt, Germany) were used for reaction monitoring. The TLC plates were either developed under iodine vapors or seen directly under UV light at 254 nm. Silica gel of 100-200 and 230-400 mesh were used for column chromatography. High resolution mass spectra were taken using Agilent 6520- Q-Tof MS/MS system. MALDI-MS spectra were taken with AB Sciex 4800 plus MALDI TOF-TOF Analyzer mass spectrometer. ^{1}H NMR spectra were recorded on Bruker Av III HD 400 MHz spectrometer operating at 400 MHz at 25 °C using 2-10 mM concentration in appropriate solvents using TMS as internal standard or the solvent signals as secondary standards and the chemical shifts (*δ)* are shown in ppm scales. Multiplicities of NMR signals are designated as s (singlet), d (doublet), t (triplet), q (quartet), br (broad), td (triplet of a doublet), dt (doublet of a triplet) and m (multiplet, for unresolved lines).

2) General experimental procedure and spectral data of compounds:

2.1 Synthesis of 6-Hydrazinonicotinic acid (2):

Hydrazine hydrate (5 mL) was added to 6-chloronicotinic acid **1** (980 mg, 6.22 mmol) and the reaction mixture was refluxed at 100 °C for 4 h. The reaction mixture was concentrated to dryness to give a solid residue. The residue was dissolved in minimum amount of water and acidified with hydrochloric acid (35 %) upto pH 5 to precipitate the HCl salt of 6-hydrazinonicotinic acid. The precipitate was filtered off, washed with ethanol to yield 800 mg (83.99 %) of 6-hydrazinonicotinic acid. ^{1}H NMR (DMSO-d_6, 400 MHz): δ/ppm = 8.53 (d, *J* = 1.72, 1H, Ar-H), 8.27 (s, 1H, NH), 7.86 (dd, *J* = 2.28, 8.88, 1H, Ar-H), 6.86 (d, *J* = 8.8, 1H, Ar-H).

2.2 Synthesis of 6-Boc-hydrazinonicotinic acid (3):

To a solution of 6-Hydrazinonicotinic acid (900 mg, 5.85 mmol) and triethylamine (0.7 mL, 5.85 mmol) in DMF (6.3 mL) was added di-*tert*- butyl dicarbonate (1.34 mL, 5.85 mmol). The reaction mixture became homogeneous over 1 h and stirring was continued for 16 h at room temperature. The reaction mixture was concentrated to dryness under reduced pressure to give a brown solid. The residue was dissolved in a minimum amount of ethyl acetate and passed through silica gel using ethyl acetate as eluant to remove coloured impurities. The eluate was concentrated to dryness to give 1.3 gm (87.75 %) of 6-Boc-hydrazinonicotinic acid which was used for next steps. ^{1}H NMR (DMSO-d_6, 400 MHz): δ/ppm = 8.99 (s, 1H, NH), 8.89 (s, 1H,

NH), 8.59 (d, *J* = 1.8, 1H, Ar-H), 7.97 (dd, *J* = 1.6, 10.88, 1H, Ar-H), 6.54 (d, *J* = 8.76, 1H, Ar-H), 1.43 (s, 9H); HRMS(ESI) calculated for $C_{11}H_{16}N_3O_4^+$ = 254.1135, found = 254.1134.

2.3 Synthesis of 42-O-(4-Formylphenylcarbonyl) rapamycin:

To a solution of 4-carboxy benzaldehyde (40 mg, 0.2 mmol) in DCM (8 mL) add DCC (41.28 mg, 0.2 mmol) at 0 °C. After 15 minutes add rapamycin (200 mg, 0.2 mmol) and DMAP (4 mg, 0.03 mmol) into it at 0 °C. The reaction was then stirred overnight at 25 °C. After that DCU was removed by filtration and washed with DCM. The organic layer was then concentrated in vacuo affording crude product. The crude residue was then purified on silica gel column, eluting with 1% MeOH/DCM to give the 100 mg (47.79 %) of title compound as a light yellow solid. Characteristic ^{1}H NMR peak ($CDCl_3$, 400 MHz): δ/ppm = 10.11(s, CHO, 1H), 8.20 (d, *J* = 8.2, Ar-H, 2H) and 7.96 (d, *J* = 8.52, Ar-H, 2H), detail NMR spectra is shown in **figure S3**; MALDI-MS $(M+Na)^+$ calculated for $C_{59}H_{83}NNaO_{15}^+$ = 1068.56, found 1068.51.

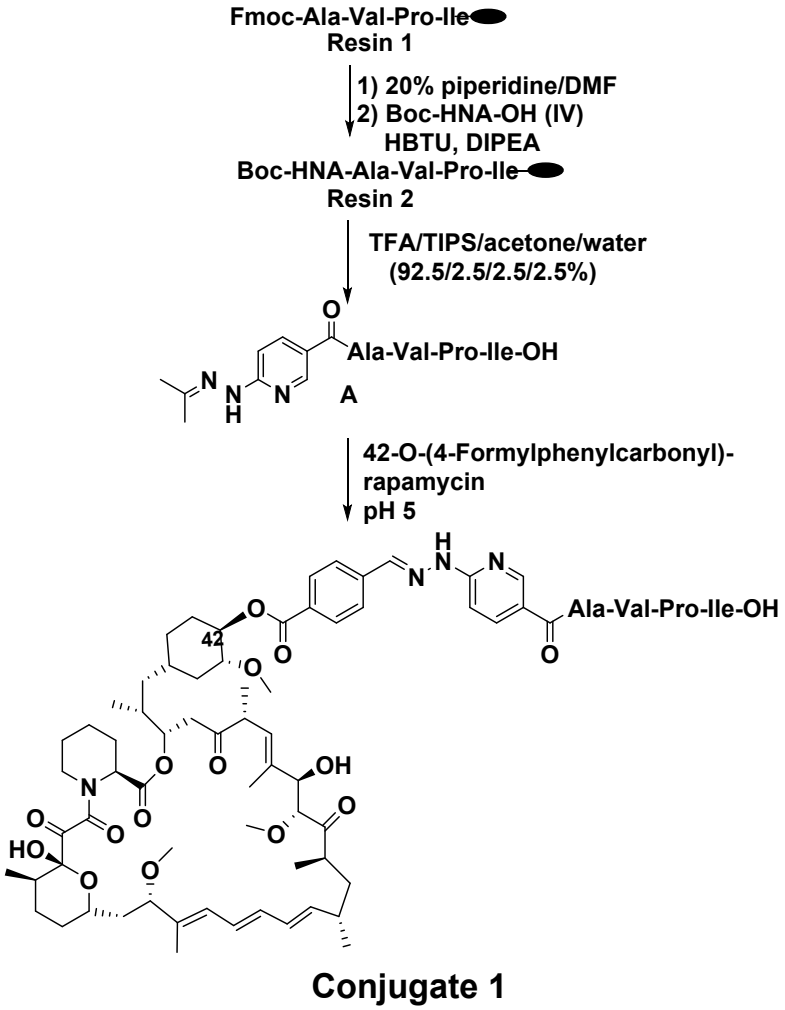
2.4 Synthesis of rapamycin-Ala-Val-Pro-Ile-OH (Conjugate 1):

*Synthesis of Isopropylidenyl-6-hydrazino nicotinoyl-Ala-Val-Pro-Ile-OH peptide (**A**):*

*Synthesis of **Resin 2:*** The resin **1** was prepared by using standard Fmoc based solid phase peptide synthesis (SPPS) on Wang resin using DIC/HOBt as a coupling reagent. After that, the resin **1** was washed with DMF (three times) and was then treated with 20% piperidine in DMF and stirred for 5 minute and filtered. The resin was again treated with 20% piperidine in DMF and stirred for 20 minutes under nitrogen and filtered. The resin was washed with DMF (three times). To this resin a solution of 6-Boc-Hydrazinonicotinic acid (3 equiv. to resin) and HBTU (3 equiv. to resin) in DMF was added and reaction mixture was stirred by purging nitrogen gas followed by addition of DIPEA (3 equiv. to HBTU) and the reaction mixture was stirred by purging nitrogen gas for 3 h under nitrogen. Sample of resin was tested for monitoring the coupling by Kaiser's test. If the test is found positive, stirring was continued till Kaiser's test is negative. After complete reaction, the resin was then washed with DMF (three times) and DCM (three times). The resin obtained was then dried under vacuum to get resin **2**.

The peptide bound resin **2** was then treated with the mixture of TFA:TIPS:acetone:water (92.5:2.5:2.5:2.5 %) and the reaction mixture was stirred for 3 h. The resin was removed by filtration and the filtrate was reduced to half by evaporation under reduced pressure below 45 °C. Peptide was precipitated with diethyl ether. Precipitate obtained was filtered and further washed three times with diethyl ether. Precipitate obtained was dried under vacuum to give the crude

peptide. Purification with RP-HPLC using C-18 column gives the desired pure peptide **A**. HRMS (ESI) $(M+H)^+$ calculated for $C_{28}H_{44}N_7O_6^+$ = 574.3348, found 574.3377.

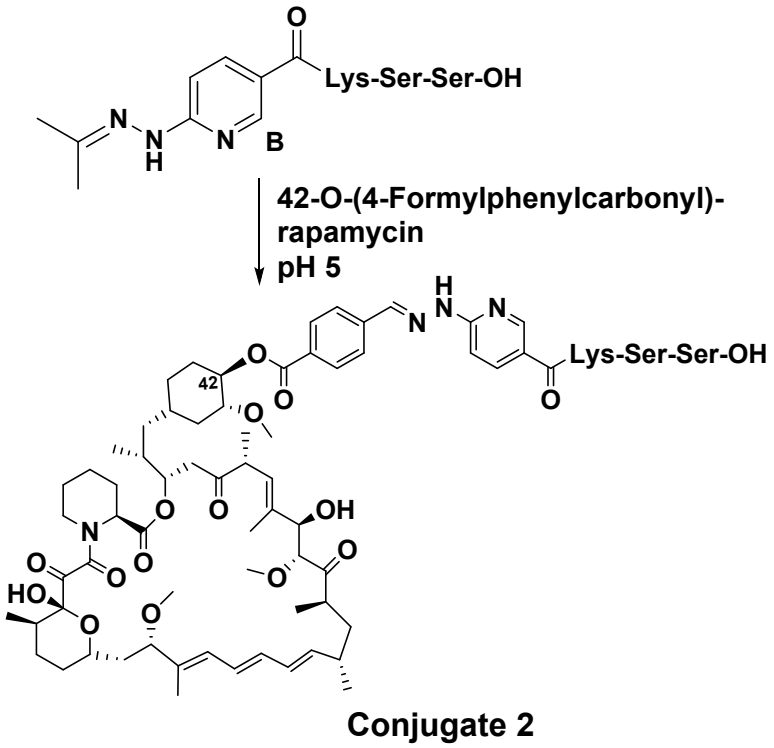


Scheme S1. Synthesis of rapamycin peptide conjugate **1**

*Synthesis of rapamycin-Ala-Val-Pro-Ile-OH (**Conjugate 1**):*

To the solution of Isopropylidenyl-6-hydrazino nicotinoyl-Ala-Val-Pro-Ile-OH (3 mg, 5 μmol) in 1 mL of sodium acetate buffer of pH 5.1 add solution of 42-O-(4-Formylphenylcarbonyl) rapamycin (5 mg, 5 μmol) in 1 mL of ethanol. Reaction was stirred for 3 h at room temperature (monitor by RP-HPLC). After that rapamycin peptide conjugate was purified by RP-HPLC in C-4 column using gradient solvent system of 0.1%TFA in DI water and acetonitrile to give conjugate **1** in almost quantitative yield. HRMS (ESI) $(M+H)^+$ calculated for $C_{84}H_{121}N_8O_{20}^+$ = 1561.8697, found 1561.9622.

2.5 Synthesis of rapamycin-Lys-Ser-Ser-OH (Conjugate 2):



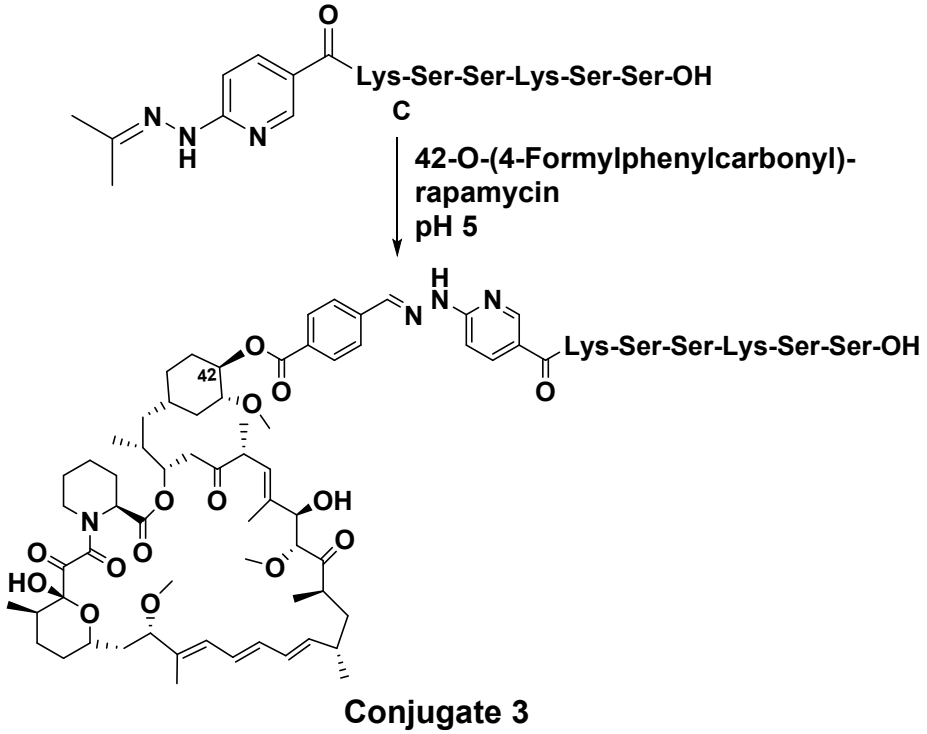
Scheme S2. Synthesis of rapamycin peptide conjugate **2**

The Isopropylidenyl-6-hydrazino nicotinoyl-Lys-Ser-Ser-OH peptide **B** is prepared by the using the same method used for the synthesis of peptide **A**. To the solution of Isopropylidenyl-6-hydrazino nicotinoyl-Lys-Ser-Ser-OH (2.5 mg, 5 μmol) in 1 mL of sodium acetate buffer of pH 5.1 add solution of 42-O-(4-Formylphenylcarbonyl) rapamycin (5 mg, 5 μmol) in 1 mL of ethanol. Reaction was stirred for 2 h at room temperature (monitor by RP-HPLC). After that rapamycin peptide conjugate was purified by RP-HPLC in C-4 column using gradient solvent system of 0.1%TFA in DI water and acetonitrile to give conjugate **2** in almost quantitative yield. HRMS (ESI) $(M+H)^+$ calculated for $C_{77}H_{111}N_8O_{21}^+$ = 1483.7858, found 1483.7735.

2.6 Synthesis of rapamycin-Lys-Ser-Ser-Lys-Ser-Ser-OH (Conjugate 3):

To the solution of Isopropylidenyl-6-hydrazino nicotinoyl-Lys-Ser-Ser-Lys-Ser-Ser-OH peptide **C** (4 mg, 5 μmol) in 1 mL of sodium acetate buffer of pH 5.1 add solution of 42-O-(4-Formylphenylcarbonyl) rapamycin (5 mg, 5 μmol) in 1 mL of ethanol. Reaction was stirred for 2 h at room temperature (monitor by RP-HPLC). After that rapamycin peptide conjugate was

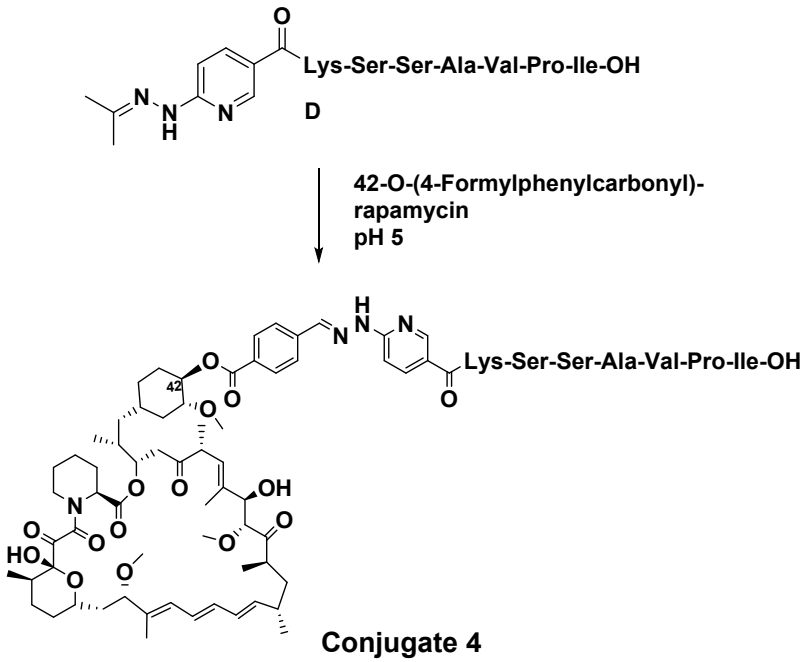
purified by RP-HPLC in C-4 column using gradient solvent system of 0.1%TFA in DI water and acetonitrile to give **3** in almost quantitative yield. HRMS (ESI) $(M+H)^+$ calculated for $C_{89}H_{133}N_{12}O_{26}^+$ = 1785.9448, found 1785.9369.



Scheme S3. Synthesis of rapamycin peptide conjugate **3**

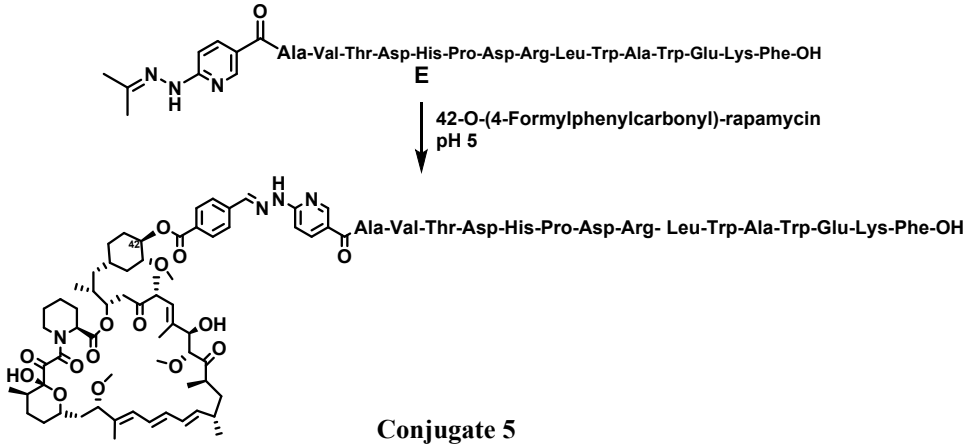
2.7 Synthesis of rapamycin -Lys-Ser-Ser-Ala-Val-Pro-Ile-OH (Conjugate 4):

To the solution of Isopropylidenyl-6-hydrazino nicotinoyl-Lys-Ser-Ser-Ala-Val-Pro-Ile-OH (4.4 mg, 5 μmol) in 1 mL of sodium acetate buffer of pH 5.1 add solution of 42-O-(4-Formylphenylcarbonyl) rapamycin (5 mg, 5 μmol) in 1 mL of ethanol. Reaction was stirred for 3 h at room temperature (monitor by RP-HPLC). After that rapamycin peptide conjugate was purified by RP-HPLC in C-4 column using gradient solvent system of 0.1%TFA in DI water and acetonitrile to give conjugate **4** in almost quantitative yield. HRMS (ESI) $(M+H)^+$ calculated for $C_{96}H_{143}N_{12}O_{25}^+$ = 1864.0282, found 1864.0287



Scheme S4. Synthesis of rapamycin peptide conjugate **4**

2.8 Synthesis of rapamycin-Ala-Val-Thr-Asp-His-Pro-Asp-Arg-Leu-Trp-Ala-Trp-Glu-Lys-Phe-OH peptide conjugate (Conjugate 5):

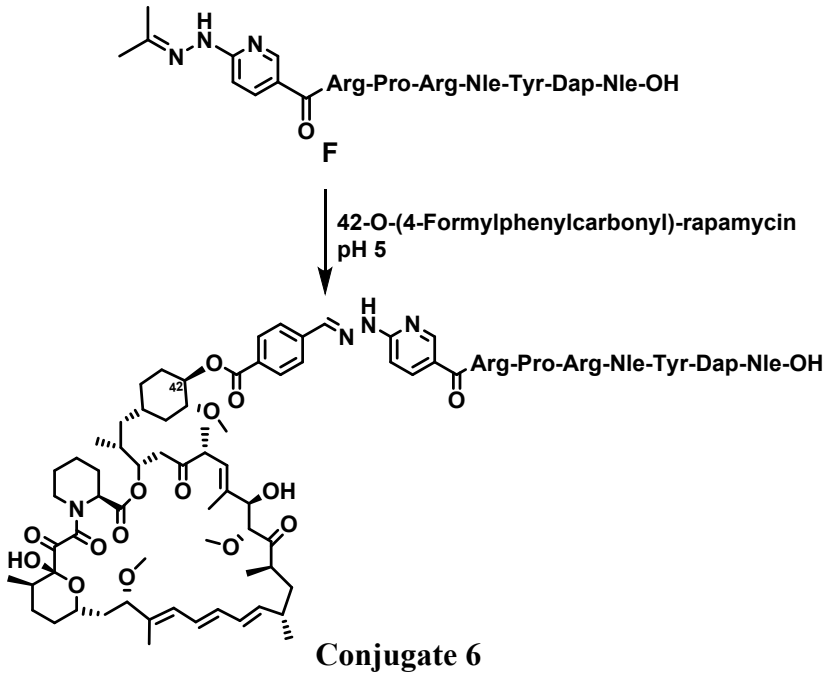


Scheme S5. Synthesis of rapamycin peptide conjugate **5**

To the solution of **E** (20 mg, 9.8 μmol) in 2 mL of sodium acetate buffer of pH 5.1 add solution of 42-O-(4-Formylphenylcarbonyl) rapamycin (10.5 mg, 9.8 μmol) in 2 mL of ethanol. Reaction was stirred for 3 h at room temperature (monitor by RP-HPLC). After that rapamycin peptide conjugate **5** was purified by RP-HPLC in C-4 column using gradient solvent system of 0.1%TFA

in DI water and acetonitrile to give 25 mg (8.3 µmol, 85 % yield) of conjugate **5** as white powder. MALDI-MS $(M+H)^+$ calculated for $C_{153}H_{210}N_{27}O_{38}^+$= 3033.53, found 3033.23.

2.9 Synthesis of rapamycin-Arg-Pro-Arg-Nle-Tyr-Dap-Nle peptide conjugate (Conjugate 6):

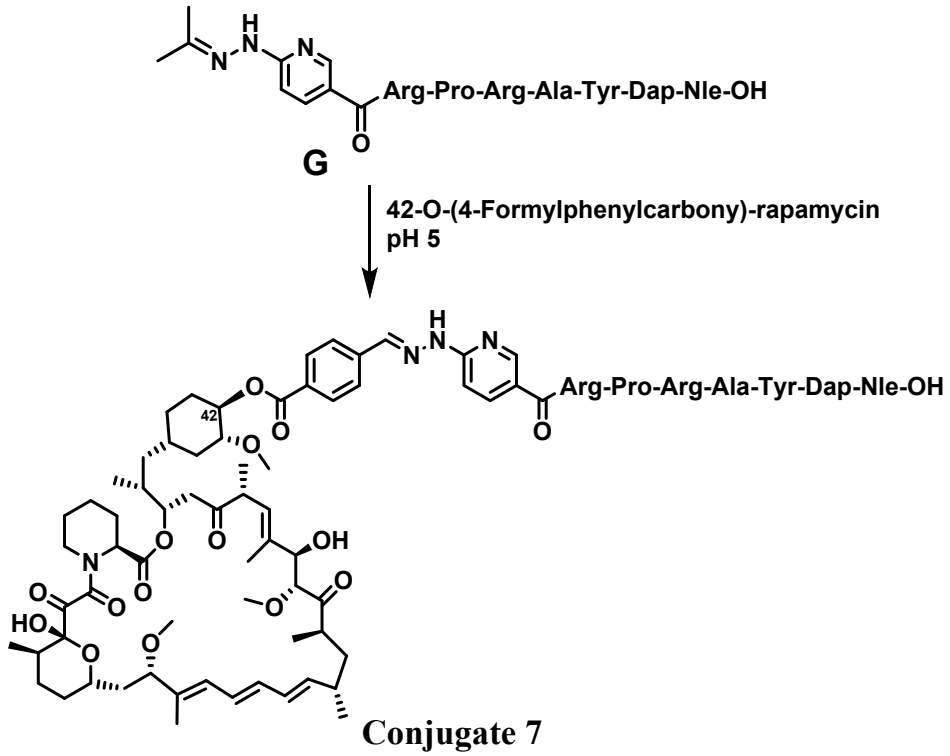


Scheme S6. Synthesis of rapamycin peptide conjugate **6**

The Isopropylidenyl-6-hydrazino nicotinoyl-Arg-Pro-Arg-Nle-Tyr-Dap-Nle (**F**) was prepared by the similar protocol as described earlier for the synthesis of **A**. To the solution of **F** (10.8 mg, 10 µmol) in 2 mL of sodium acetate buffer of pH 5.1 add solution of 42-O-(4-Formylphenylcarbonyl) rapamycin (10.5 mg, 10 µmol) in 2 mL of ethanol. Reaction was stirred for 3 h at room temperature (monitor by RP-HPLC). After that rapamycin peptide conjugate **6** was purified by RP-HPLC in C-4 column using gradient solvent system of 0.1%TFA in DI water and acetonitrile to give 18 mg (8.7 µmol, 87 % yield) of conjugate **6** as white powder. MALDI-MS $(M+H)^+$ calculated for $C_{106}H_{157}N_{18}O_{24}^+$= 2066.16, found 2066.12.

2.10 Synthesis of rapamycin-Arg-Pro-Arg-Ala-Tyr-Dap-Nle peptide conjugate (Conjugate 7):

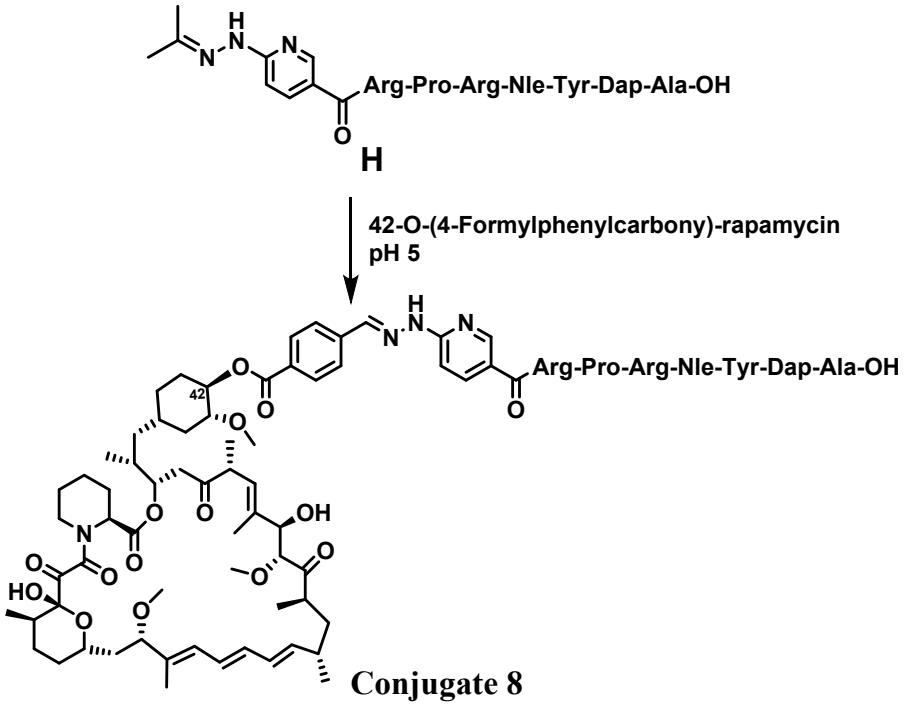
The isopropylidenyl-hydrazino nicotinoyl-Arg-Pro-Arg-Ala-Tyr-Dap-Nle peptide (**G**) required for the synthesis of conjugate **7** was prepared by using similar protocol described in the earlier sections. To the solution of **G** (10.4 mg, 10 µmol) in 2 mL of sodium acetate buffer of pH 5 add solution of 42-O-(4-Formylphenylcarbonyl) rapamycin (10.5 mg, 10 µmol) in 2 mL of ethanol. Reaction was stirred for 3 h at room temperature (monitor by RP-HPLC). After that rapamycin peptide conjugate **7** was purified by RP-HPLC in C-4 column using gradient solvent system of 0.1%TFA in DI water and acetonitrile to give 19 mg (9.4 µmol, 94 % yield) of conjugate **7** as white powder. MALDI-MS $(M+H)^+$ calculated for $C_{103}H_{151}N_{18}O_{24}^+$= 2024.11, found 2023.91.



Scheme S7. Synthesis of rapamycin peptide conjugate **7**

2.11 Synthesis of rapamycin-Arg-Pro-Arg-Nle-Tyr-Dap-Ala peptide conjugate (Conjugate 8):

The required isopropylidenyl-hydrazino-nicotinoyl-Arg-Pro-Arg-Nle-Tyr-Dap-Ala peptide **H** was prepared separately by the same method as described in the earlier sections. To the solution of hydrazine-nicotinoyl tagged peptide **H** (10.4 mg, 10 µmol) in 2 mL of sodium acetate buffer of pH 5.1 add solution of 42-O-(4-Formylphenylcarbonyl) rapamycin (10.5 mg, 10 µmol) in 2 mL of ethanol. Reaction was stirred for 3 h at room temperature (monitor by RP-HPLC). After that rapamycin peptide conjugate **8** was purified by RP-HPLC in C-4 column using gradient solvent system of 0.1%TFA in DI water and acetonitrile to give 18 mg (8.9 µmol, 89 % yield) of conjugate **8** as white powder. MALDI-MS $(M+H)^+$ calculated for $C_{103}H_{151}N_{18}O_{24}^+$= 2024.11, found 2024.08.



Scheme S8. Synthesis of rapamycin peptide conjugate **8**

3) Copies of ^{1}H NMR and Mass spectra:

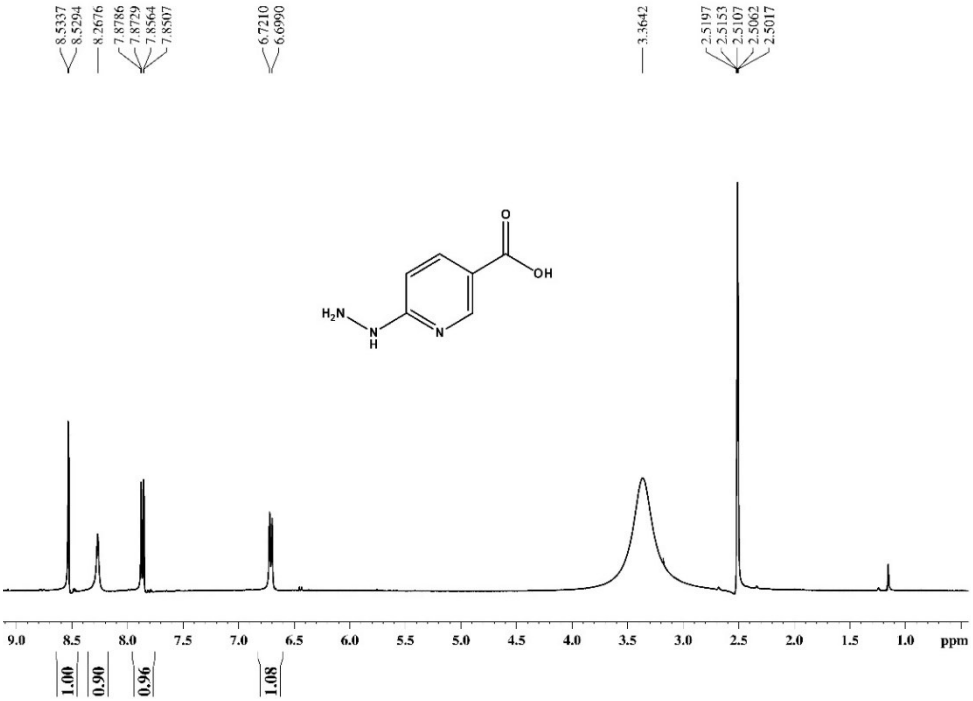


Figure S1: ^{1}H NMR spectrum of compound **2** (DMSO-d_6, 400 MHz).

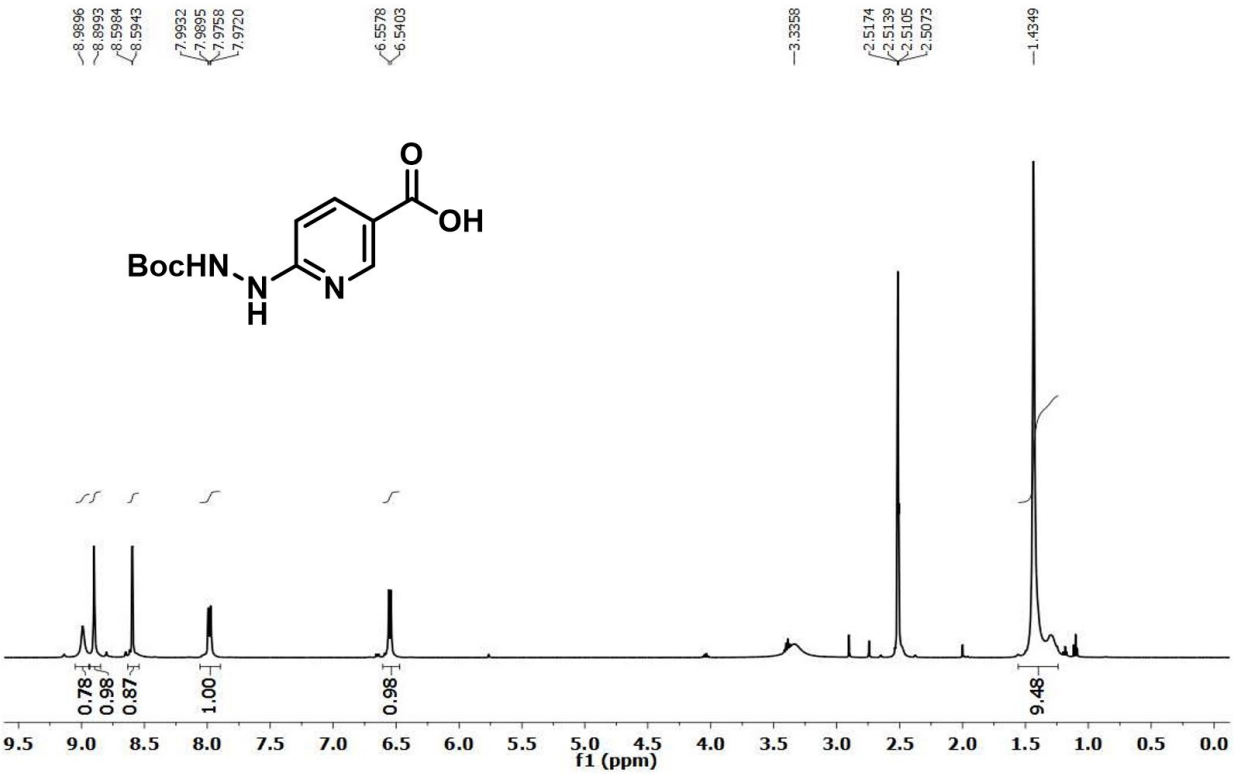


Figure S2: ^{1}H NMR spectrum of compound **3** (DMSO-d_6, 500 MHz).

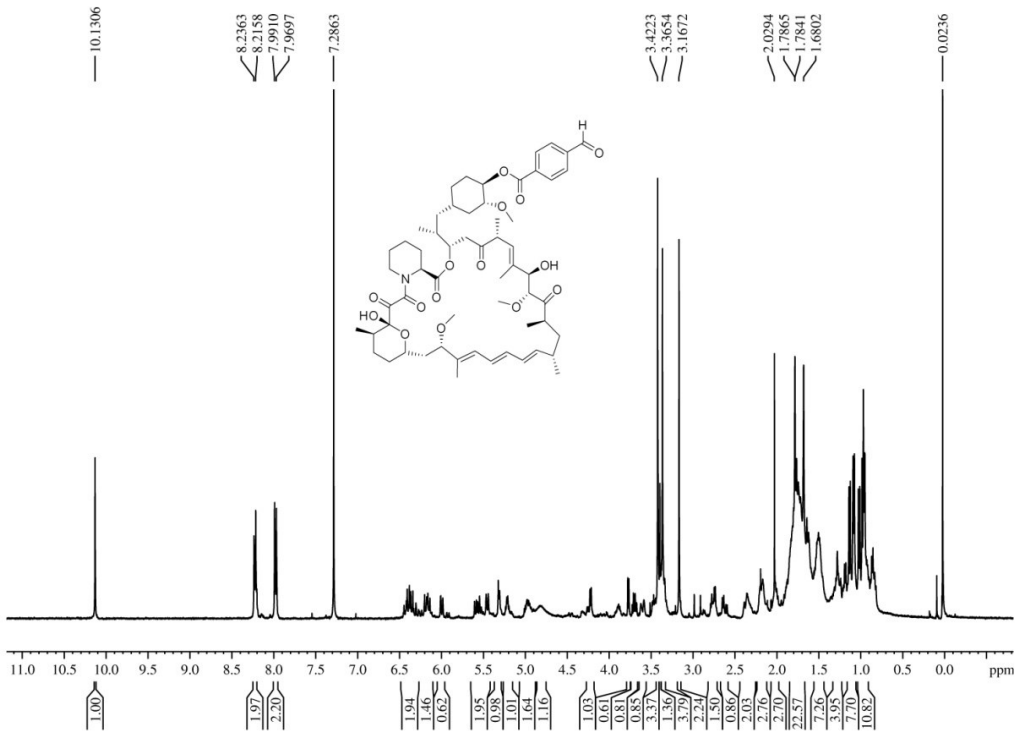


Figure S3: ^{1}H NMR spectrum of 42-O-(4-Formylphenylcarbonyl) rapamycin ($CDCl_3$, 400 MHz)

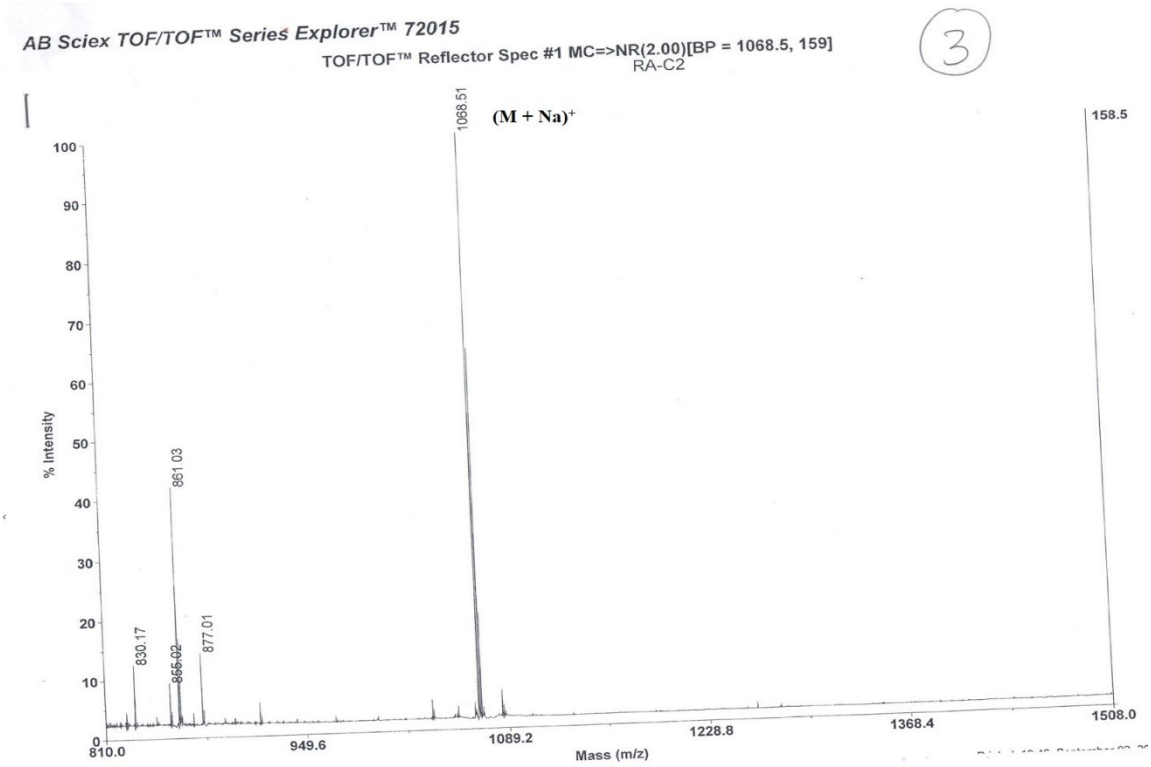


Figure S4: MALD-MS spectrum of 42-O-(4-Formylphenylcarbonyl) rapamycin.

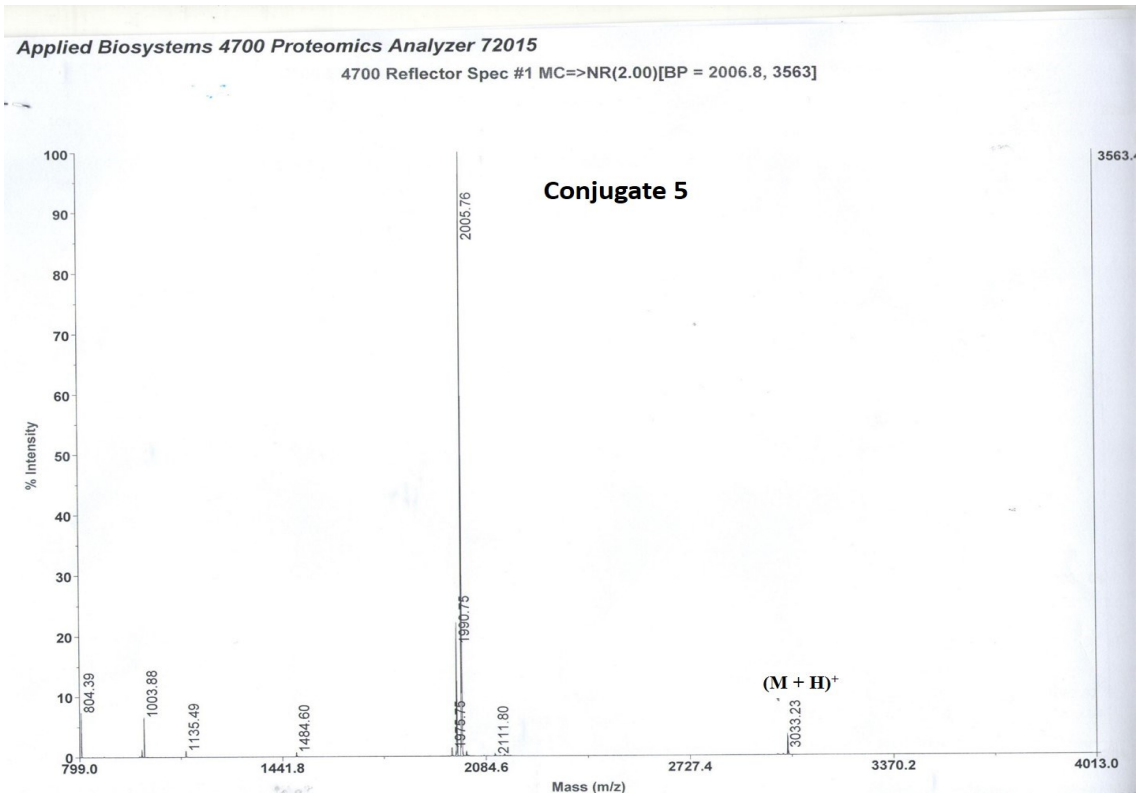


Figure S5: MALDI-MS spectrum of Conjugate **5**.

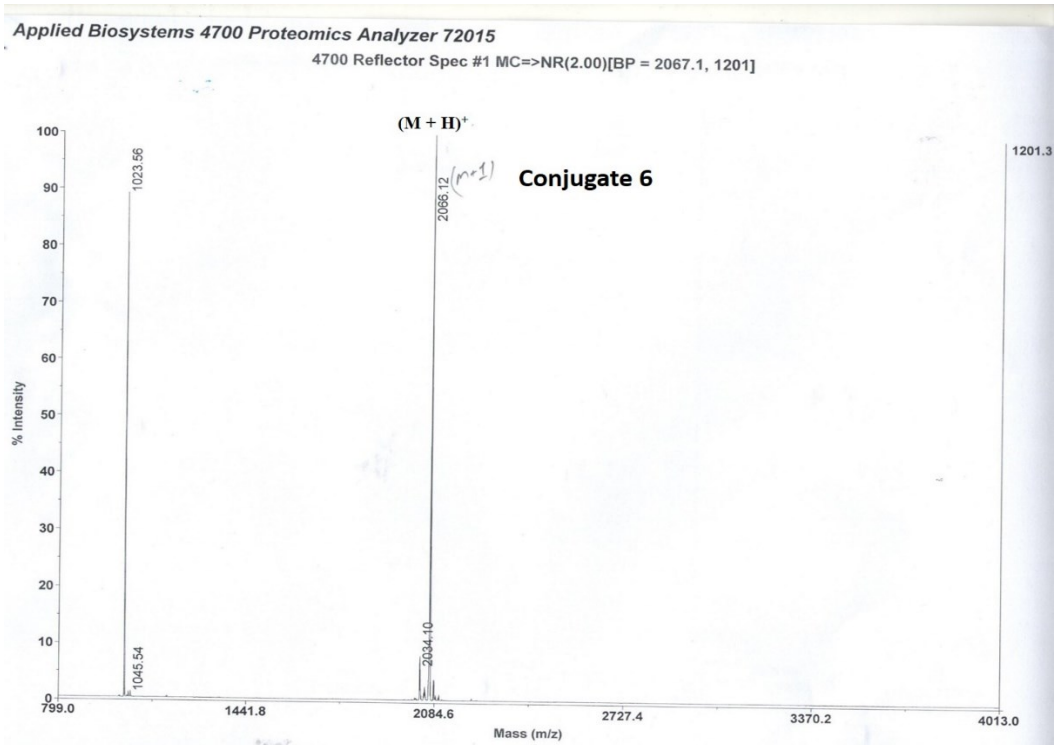


Figure S6: MALDI-MS spectrum of Conjugate **6**.

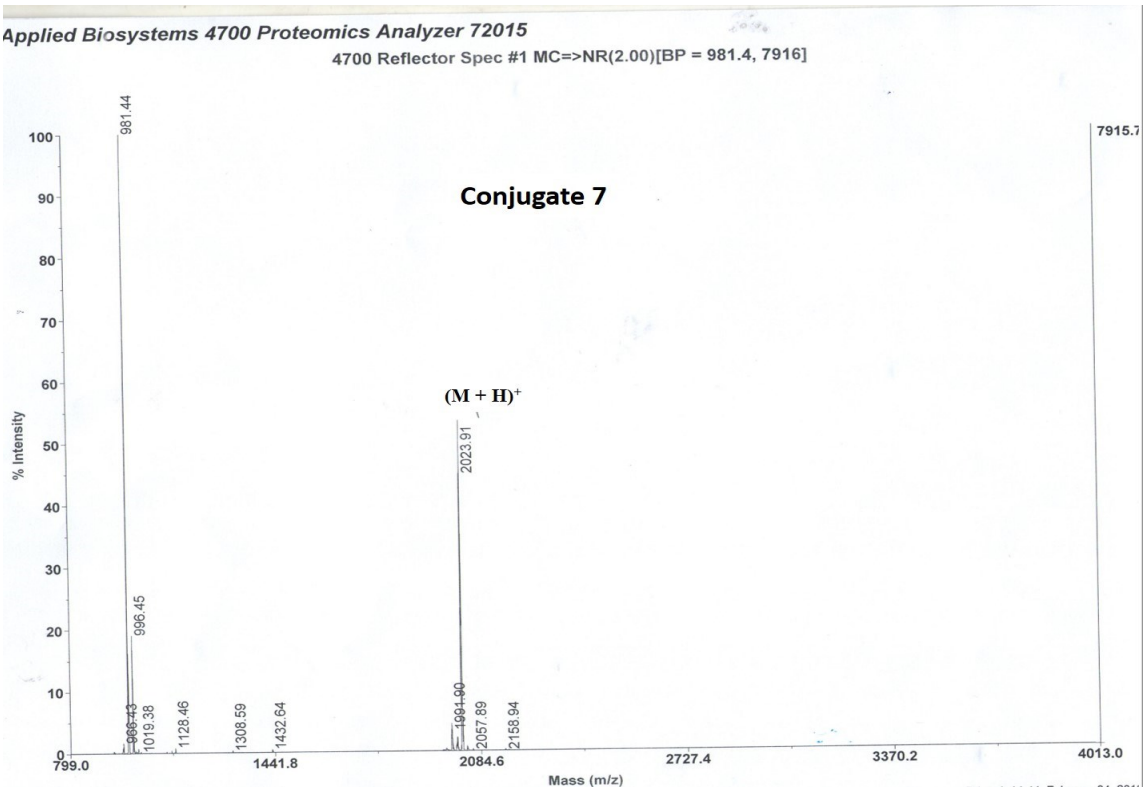


Figure S7 MALDI-MS spectrum of Conjugate **7**.

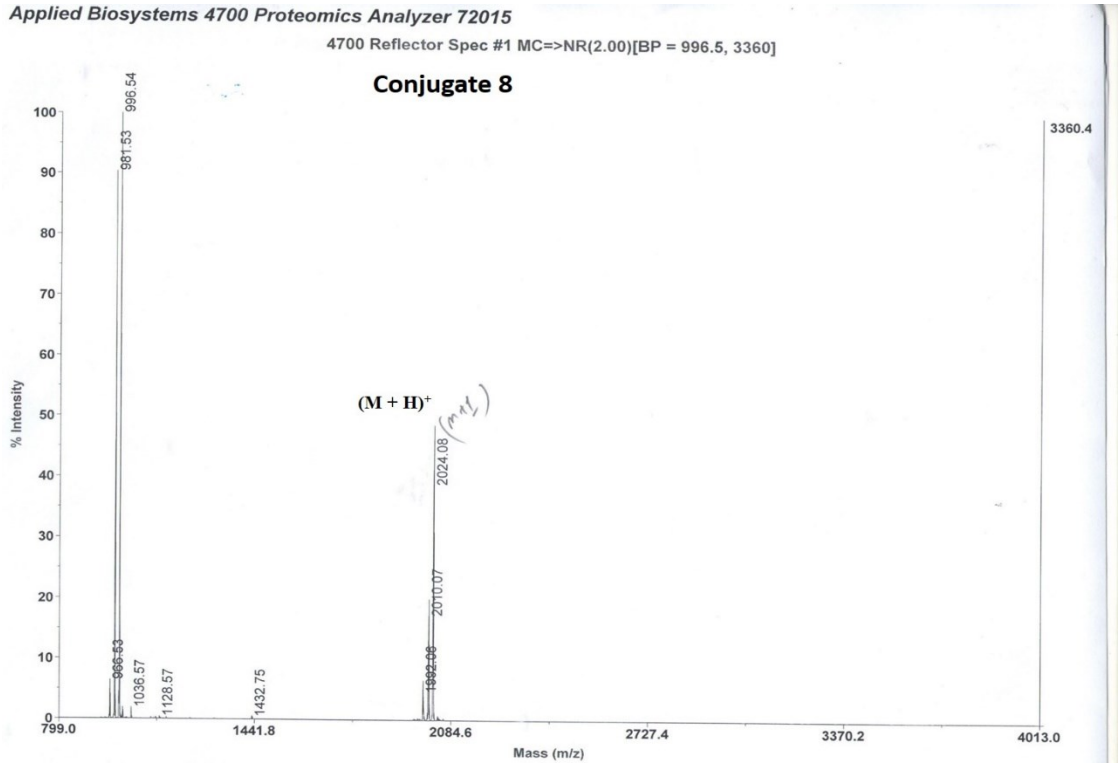
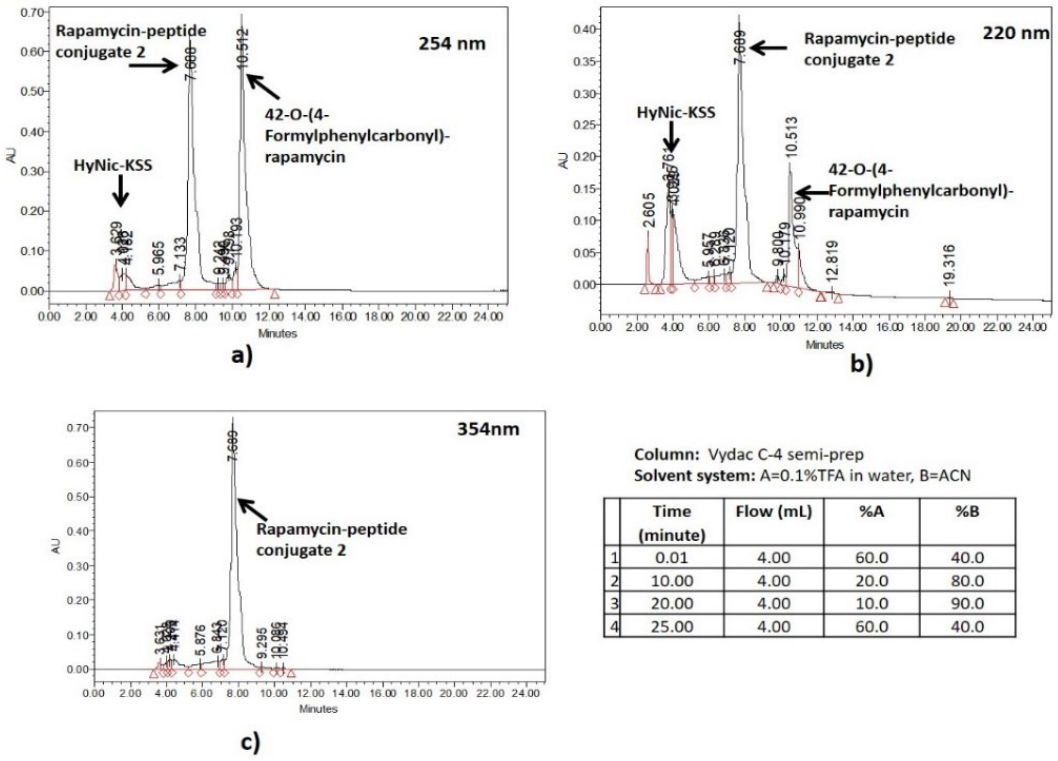


Figure S8: MALDI-MS spectrum of Conjugate **8**.

4) Traces of HPLC spectra of rapamycin peptide conjugates:

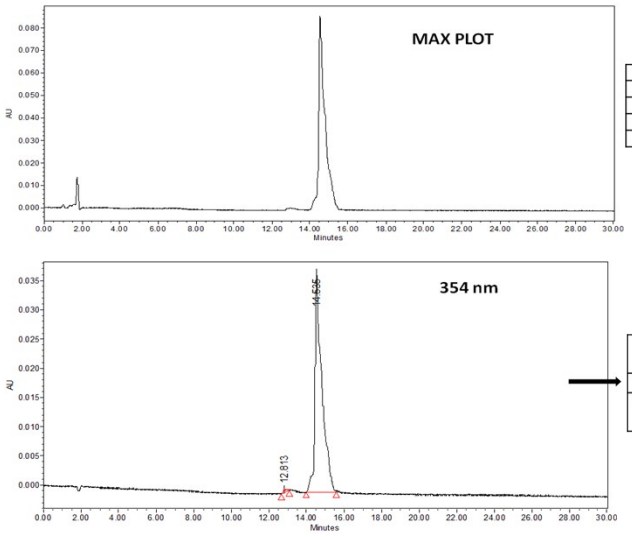
The HPLC chromatograms of the conjugate **2**, **5, 6, 7 and 8** are shown in figure **S9-S13**. The chromatogram is recorded also at 354 nm to show the characteristic absorption of bis-aryl hydrazone chromophore suggesting that conjugates have bis-aryl hydrazone linkers in their structure.



Column: Vydac C-4 semi-prep
Solvent system: A=0.1%TFA in water, B=ACN

	Time (minute)	Flow (mL)	%A	%B
1	0.01	4.00	60.0	40.0
2	10.00	4.00	20.0	80.0
3	20.00	4.00	10.0	90.0
4	25.00	4.00	60.0	40.0

Figure S9: HPLC spectra of crude reaction mixture of conjugation reaction between Isopropylidenyl-6-hydrazino nicotinoyl-Lys-Ser-Ser-OH peptide (**HyNic-KSS**) and 42-O-(4-Formylphenylcarbonyl)-rapamycin at different wavelength. The product (**Conjugate 2**) was easily identified at 354 nm, which is correspond to bis aryl hydrazone bond formation in product.

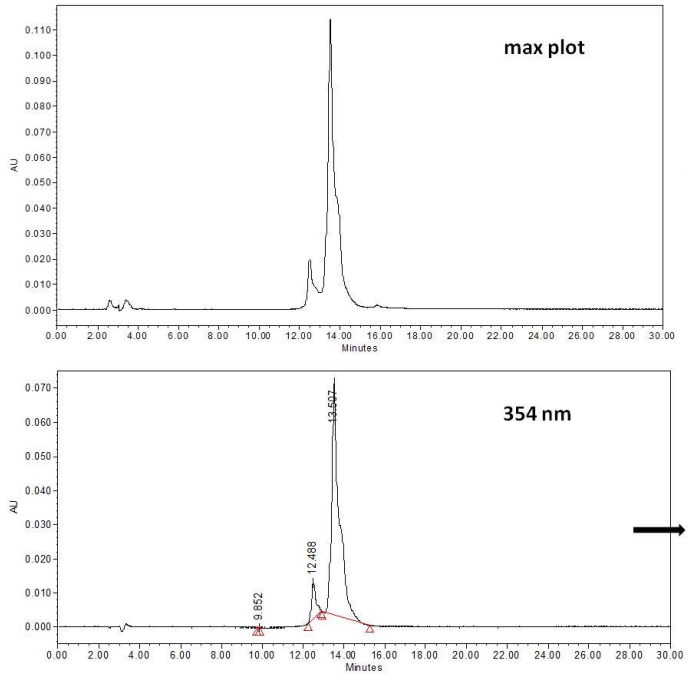


Column : XBridge BEH, C-18 (4.6 x 150 mm, 5µ)
Solvent system: A = 0.1 % TFA in water, B = ACN

Time (min.)	Flow (mL)	%A	%B
0.01	1.00	70.0	30.0
10.00	1.00	30.0	70.0
25.00	1.00	5.0	95.0
30.00	1.00	70.0	30.0

	Retention Time	Area	% Area	Height
1	12.813	8110	0.83	599
2	14.535	965492	99.17	37401

Figure S10: HPLC spectra of conjugate **5**

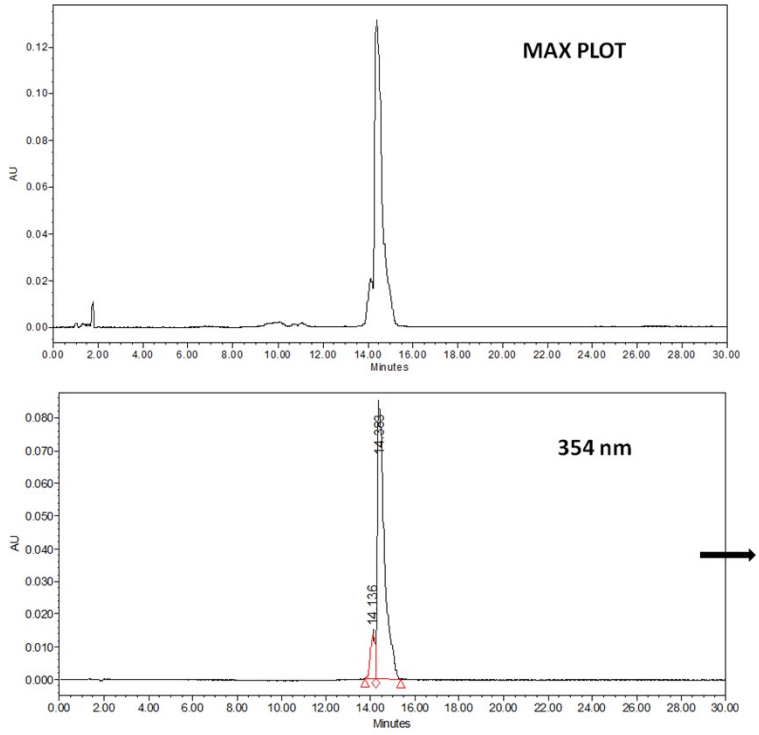


Column : C-4 VYDAC 4.6 X 250 mm
Solvent system: A = 0.1 % TFA in water, B = ACN

Time (min.)	Flow (mL)	%A	%B
0.01	1.00	70.0	30.0
10.00	1.00	30.0	70.0
25.00	1.00	5.0	95.0
30.00	1.00	70.0	30.0

	Retention Time	Area	% Area	Height
1	9.852	328	0.02	-105
2	12.488	152653	7.90	10484
3	13.507	1780050	**92.09**	67797

Figure S11: HPLC spectra of conjugate **6**

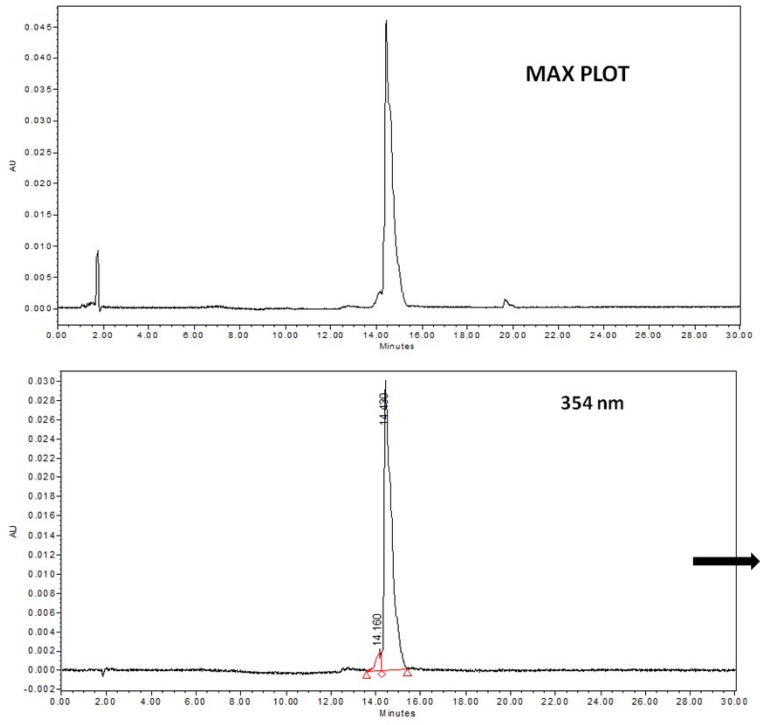


Column : XBridge BEH, C-18 (4.6 x 150 mm, 5µ)
Solvent system: A = 0.1 % TFA in water, B = ACN

Time (min.)	Flow (mL)	%A	%B
0.01	1.00	70.0	30.0
10.00	1.00	30.0	70.0
25.00	1.00	5.0	95.0
30.00	1.00	70.0	30.0

	Retention Time	Area	% Area	Height
1	14.136	187507	9.02	13590
2	14.383	1891899	90.98	83279

Figure S12: HPLC spectra of conjugate **7**



Column : XBridge BEH, C-18 (4.6 x 150 mm, 5µ)
Solvent system: A = 0.1 % TFA in water, B = ACN

Time (min.)	Flow (mL)	%A	%B
0.01	1.00	70.0	30.0
10.00	1.00	30.0	70.0
25.00	1.00	5.0	95.0
30.00	1.00	70.0	30.0

	Retention Time	Area	% Area	Height
1	14.160	31149	4.42	1862
2	14.430	673070	**95.58**	29493

Figure S13: HPLC spectra of conjugate **8**

5) In vitro mTOR kinase inhibition assay:

Antibodies and reagents

Antibodies to p-Ser^{473}Akt, Total Akt, T^{389}p70S6K, Total p70S6K were purchased from Cell Signaling Technology (Beverly, MA; USA). HRP-conjugated goat anti-rabbit was procured from Jackson Immuno Research Europe Ltd. (UK). Protein A/G sepharose beads were purchased from BioVision Inc (Cara ct, PA, USA). Rapamycin (553210-100UGCN), active recombinant mTOR protein and FKBP12 kit (cat. #14-784) was procured from Merck Millipore. mTOR kinase buffer (PV4794) was purchased from Life Sciences,USA. Torin-1 was purchased from Tocris (cat. 4247).

Cell culture and treatment

MCF-7 cells were obtained from ATCC (Manassas, VA, USA). MCF-7 cells were cultured in RPMI 1640 media supplemented with antibiotic and 10% FBS. All cells were maintained in a humidified atmosphere (95% humidity) at 37º C and 5% CO_2.

Western blotting

The expression level of various proteins was evaluated using Western blotting. Immunoprecipitated protein samples were resolved through SDS-PAGE and were transferred to PVDF membranes. After blocking with 5% BSA in TBST, membranes were incubated at 4 ºC overnight with specific antibodies. Next day, blots were incubated with horseradish peroxidase-conjugated secondary antibody. Blots were developed with the ECL chemiluminescence substrate on the X-Ray film manually.

Cell lysis and immunoprecipitation-based isolation of mTORC2 and AKT

The in vitro mTOR kinase assay was carried out using mTORC2 isolated from MCF-7 cells and Active Recombinant mTOR Protein (merck). Isolation of mTORC2 was done according to Huang et.al. with slight modifications.[1, 2] Briefly, the cells were harvested after 15 minutes of insulin treatment (100 nM) and lysed in non-denaturing CHAPS lysis buffer (40 mM HEPES of pH 7.4, 120 mM NaCl, 2 mM EDTA, 0.3% CHAPS, 10 mM pyrophosphate, 10 mM glycerophosphate, 50 mM NaF) containing protease inhibitor cocktail.[3] The lysates were centrifuged at 16,500 g for 10 min at 4 ºC and supernatant was utilized for isolation of mTORC2 using anti-Rictor rabbit monoclonal antibody directed co-immunoprecipitation. Subsequently, protein G beads containing mTORC2 immunocomplexes were washed four times with CHAPS lysis buffer at 6000g for 1 min at 4 ºC, followed by a single wash with mTORC2 kinase buffer

containing 25 mM HEPES of pH 7.4, 100 mM potassium acetate, and 1 mM $MnCl_2$, and utilized for in vitro kinase assay.

Isolation of AKT and p70S6K (to serve as substrate) was also carried out through anti-AKT rabbit monoclonal antibody and anti-p70S6K rabbit polyclonal antibody directed immunoprecipitation from a whole cell lysate of MCF-7 cells that were switched to CS-FBS containing media for 24 hrs prior to harvest. Furthermore, to ensure that immunoprecipitated AKT and p70S6K remained dephosphorylated at Ser^{473} and Thr^{389} consecutively to serve as a substrate during the kinase reaction, 2 hrs prior to harvesting, the cells were treated with Wortmannin (10 μM), a potent PI3K selective inhibitor. The protein G beads containing immunoprecipitated AKT and protein A beads containing immunoprecipitated p70S6K was then eluted by soft elution method[4] and resuspended in mTORC2 kinase buffer to utilized as a substrate during in vitro kinase assay.

Kinase Assay and Immunoblot Analysis

The mTORC2 and AKT immunocomplexes isolated as described above were utilized for in vitro kinase assay.[5,6] For in vitro kinase assay firstly mTOR inhibitor was done using Conjugates (10 nM), Rapamycin (1 nM, 10 nM) and Torin-1 (10 nM) followed by kinase assay. FKBP12 (13ug/ml) and rapamycin (1 nM, 10 nM) was incubated for 5 min on ice for rapamycin activation prior to mTOR inhibition. Inhibitors were incubated with Active mTOR recombinant protein (~40 ng) and the protein G beads immunocomplexes of mTORC2 (~40 ng) for 30 min on ice for mTOR inhibition. Inhibited mTOR's, p70S6K (~6.5 μg) and AKT (~6.5 μg) were then equilibrated in mTORC2 kinase buffer for 5 min on ice. A parallel reaction mixture containing all the components except for mTOR and its substrates (p70S6K and AKT) was considered as blank, also the reaction mixtures containing all the components except for mTOR and other with mTOR but without substrates (p70S6K and AKT) was considered as negative controls. The reaction was initiated by addition of ATP (100 μM) and transferring the tubes at 30 ºC in shaking water bath at 100 rpm for 2 hrs. The reaction was terminated by adding 10 mM EDTA for 5 mins at room temperature. Subsequently, the reaction mixture was denatured by adding an equal volume of sample buffer followed by boiling for 5 mins. Thereafter, aliquots of the reaction mixture were resolved through 12% denaturing polyacrylamide gel, followed by Western blotting for detecting the levels of p-Ser^{473}AKT and p-The^{389}p70S6K.

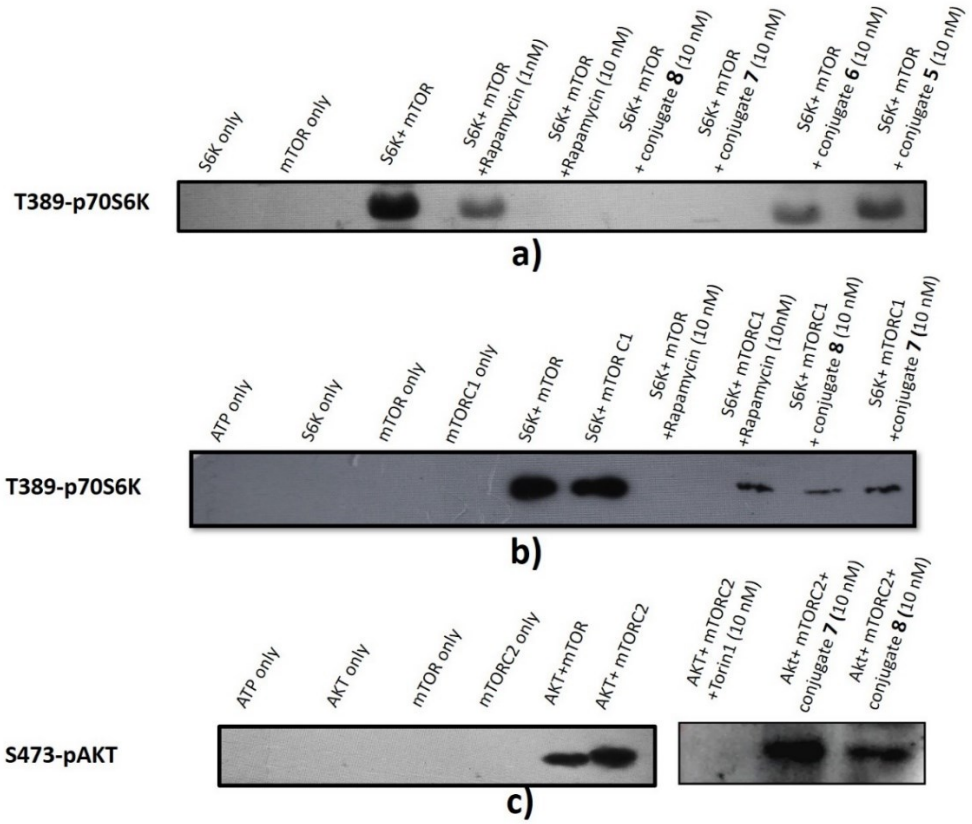


Figure S14: The *in-vitro* inhibition of kinase activity of recombinant mTOR protein, mTORcomplex1 and mTOR complex2 isolated from MCF-7 breast cancer cell line. Western blot showing inhibition of kinase activity of; a) recombinant mTOR protein; b) mTOR comp complex 1 and c) mTOR complex 2. In case of mTORC2 Torin 1 is used as standard inhibitor of kinase activity of mTORC2.

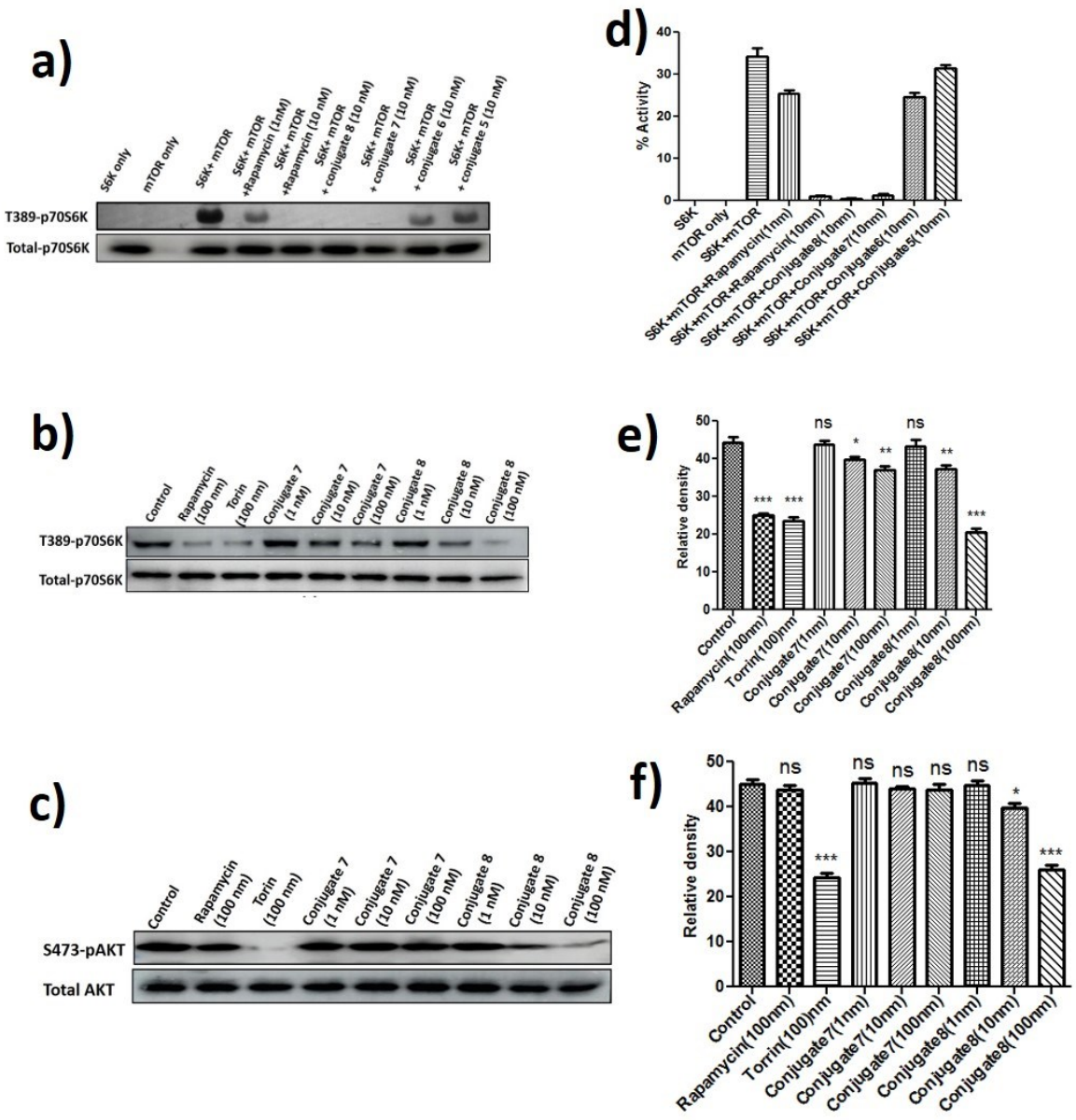


Figure S15: The in-vitro inhibition of kinase activity of recombinant mTOR protein, mTORcomplex1 and mTOR complex2 in MCF-7 breast cancer cell line. Western blot showing inhibition of kinase activity of; a) recombinant mTOR protein; b) mTOR complex 1 in MCF-7 cells and c) mTOR complex 2 in mCF-7 cells. Torin is used as standard inhibitor of kinase activity of mTORC1 and mTORC2. Quantification (by densitometric analysis) of phosphorylation inhibition of protein d) S6K by mTOR; e) S6K by mTORC1 and f) AKT by mTORC2

6) Akt kinase inhibition assay:

The optimized non-radioactive *in-vitro* ELISA based Akt kinase assay (through binding at ATP binding site), was performed.[7] In short ELISA plates were coated with Akt substrate (purified GSK-3 fusion protein). Kinase activity was performed at 30 °C after adding active Akt and ATP. Phosphorylated GSK-3 fusion protein was measured colorimetrically after incubation with appropriate primary and secondary antibodies. A443654 a known Akt inhibitor is used as positive control.[8]

7) Cell based assay:

We next sought to evaluate cellular activity of these conjugates in two breast cancer cell lines using well established SRB (Sulphorodamine B) assay [9,10] which is described as follows.

Cell Culture

Human Breast cancer cell line including MCF-7 & MDA-MB-231 were obtained from ATCC, USA. We used early passage cells and cultured in their appropriate medium. The enumerated cells are dispensed in a 96-well tissue culture plate. Each well receives 100 µl of the cell suspension containing 10,000-15000 cells (depending upon the nature of cell line). The cells are then incubated at 37 ºC in 5% CO_2 concentration for 24 h before addition of the drugs.

Addition of test samples: 100 µl of working solution of the test sample is added to the cell monolayer to give a final concentration 10 µM (pure compound) For each sample, duplicate wells are included.

Addition of SRB and Colorimetric reading: After 48 h incubation, cells attached to substratum of the plate are fixed by adding cold 50% trichloroacetic acid (TCA, 50 µl/well) on top of the medium and incubated at 4 ºC for 1 h. After that the plate is gently washed 5 times with slow running tap water via plastic tubing to remove TCA, culture medium and dead cells. After washing, the plates are allowed to dry in air after fixing and drying step) 50 µl/well of SRB solution is added and left at room temperature for 30 min. At the end of the staining period, unbound SRB is removed by quickly rinsing plates 4-5 times with 1 % (v/v) acetic acid. Plates are allowed to air-dry at room temperature then 150 µl of 10 mM Tris base solution is added to each well and plate is shaken for 15 min on a gyratory shaker to solubilize the protein-bound dye. Alternatively, if a shaker is unavailable, SRB gets solubilized after 30 min in Tris base solution. Absorbance was measured on a plate reader (epoch microplate reader, biotek) at 510 nm to calculate the percent inhibition in cell growth by using the formula: [100(absorbance of compound treated cells/absorbance of un-treated cells)] 100.

Table S1 Percentage inhibition of conjugates in two different breast cancer cell lines having different sensitivity towards rapamycin at 10μM concentration

S.No.	Compounds	MDA-MB-468	MDA-MB-231
1.	Rapamycin	53.04	15.73
2.	**Conjugate 1**	45.23	-10.04
3.	**Conjugate 2**	42.37	-0.57
4.	**Conjugate 3**	43.27	-12.25
5.	**Conjugate 4**	47.31	1.17

Table S2 Percentage inhibition of conjugates in MDA-MB-468 cell lines at 10, 1, 0.5 and 0.25 μM concentration

S.No.	Compounds	10 μM	1 μM	0.5 μM	0.25 μM
1.	Rapamycin	53.04	43.27	45.12	34.67
2.	**Conjugate 1**	45.23	39.61	39.31	34.45
3.	**Conjugate 2**	42.37	38.48	33.39	29.13
4.	**Conjugate 3**	43.27	41.48	40.90	34.36
5.	**Conjugate 4**	47.31	43.76	41.12	36.14

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