Electronic Supplementary Information (ESI)

Characterization of Mammalian N-degrons and Development of Heterovalent Inhibitors of the N-end Rule Pathway

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ESI Methods

In vitro degradation/ubiquitination assay.

Time- and concentration-dependent *in vitro* degradation assays were performed as described.[1] Briefly, biotinylated lysine-tRNA complex (Transcend tRNA, Promega) was added to a reaction mixture to randomly label DHFR-Ub-X-nsP4 fusion protein with biotin. The nonstructural protein 4 (nsP4) of Sindbis virus originally bears N-terminal Tyr and it is degraded by the N-end rule pathway[2,3]. The N-terminal residue of nsP4 is mutagenated to various amino acids (X-nsP4) to generate N-end rule substrates and controls. Biotinylated proteins were detected with horseradish peroxidase (HRP)-conjugated streptavidin (Pierce). For quantitative immunoblotting, proteins were visualized by infrared fluorescence using the Odyssey Imaging System and IR dye-conjugated streptavidin (Li-COR). For autoradiographic assay, ^{35}S-methionine (Amersham Bioscience) and supplements were added to the *in vitro* transcription/translation reaction. To produce the DHFR-Ub-X-nsP4 plasmids, X-nsP4 was amplified by PCR and subcloned into the UPR vector pcDNA3(dEheI)FDHUMCM using *Sma*I/*Xba*I cut. Ubiquitination of test proteins was characterized in the presence of 5 μM MG132, followed by anti-Ub immunoprecipitation and anti-biotin Western blotting

Mammalian Cell Culture and transient expression.

HeLa cells were grown in DMEM supplemented with 10 % FBS, 2 mM glutamine, and 100 units/ml penicillin/streptomycin. Cells were transfected with 2-4 μg of plasmid DNA expressing X-GFP in a 6-well culture plate for 4 hr using LipofectAMINE 2000 (Invitrogen) when cells were >95% confluent or at a density of 10^6 cells/well. Cell lysates were prepared 36-48 hr post-transfection in RIPA buffer and used for immunoblotting. In a given panel, each lane is loaded with extract from an equal cell number, generally corresponding to 10-20 μg/lane, or 1/10 of the sample recovered from one well of a 6-well plate.

Expression of N-end rule model substrates in mammalian cells.

Mammalian cells, such as MEF and cardiac cells, were transiently transfected with 2~4 μg of

total plasmid DNA in a 6-well plate for 4~6 hr using Lipofectamine2000 (Invitrogen) when cells were >95% confluent. Cell lysates were collected ~36 hr post-transfection in RIPA buffer and used for immunoblotting or immunoprecipitation. The level of total ubiquitinated Arg-GFP was detect in the presence of 10 μM MG132. For pulse-chase analysis, cells were labeled with ^{35}S -methionine/cysteine (^{35}S-Express protein labeling mix; Perkin-Elmer) after 24 hr of transfection, followed by a cycloheximide chase. Whole cell extracts were prepared and immunoprecipitated, followed by SDS-PAGE and autoradiography, as previously described.[4,5]

***In silico* docking analysis of protein-protein and protein-ligand interactions.**

Crystal structures of UBR1, UBR2, and ClpS boxes were taken from the Protein Data Bank (PDB ID: 3NY1, 3NY3, and 3DNJ, respectively). Because the "combined" UBR2-ClpS structure is unknown, the Gramm-X protein-protein docking web server[6] was used to dock the UBR2 and ClpS boxes. Two possible UBR2-ClpS structures were found with most stable binding energies. The RIFS and YLFV peptide structures were extracted from their complexes with the UBR2 box and ClpS, respectively. Other peptides were created with the Mutagenesis module in the PyMOL package (http://sourceforge.net/projects/pymol/). The RF-C5 structure was optimized using the semi-empirical PM6 method[7] with the Gaussian 09 program.[8]

AutoDockTools[9] version 1.5.4 was used to add polar hydrogens and assign Gasteiger charges[10] to proteins, peptides, and RF-C5. For each protein, AutoGrid version 4.2 was used to create affinity grids centered on the active site. The grids were large enough to contain all the active sites. AutoDock version 4.2 with the Lamarckian genetic algorithm was used to simulate ligand-receptor docking.[11] Docking parameters were as follows: trials of 200 dockings, population size of 300, random starting position and conformation, translation step ranges of 2.0 Å, rotation step ranges of 50°, elitism of 1, mutation rate of 0.02, cross over rate of 0.8, local search rate of 0.06, and 50 million energy evaluations. Docked conformations were clustered using a tolerance of 2.0 Å RMSD. The lowest binding energy of the most populated cluster was compared to experimental data. Experimental binding free energies were calculated from dissociation constants based on the equation: $\Delta G = RT \ln K_d$, where R is gas constant (1.987 cal K^{-1} mol^{-1}) and T is temperature (298.15 K). The PBD2PQR web server[12] was used to create a PQR file at pH=7 and AMBER force field.[13] Electrostatic

potentials were calculated using APBS package.[14] All structural figures were prepared using the PyMOL package.

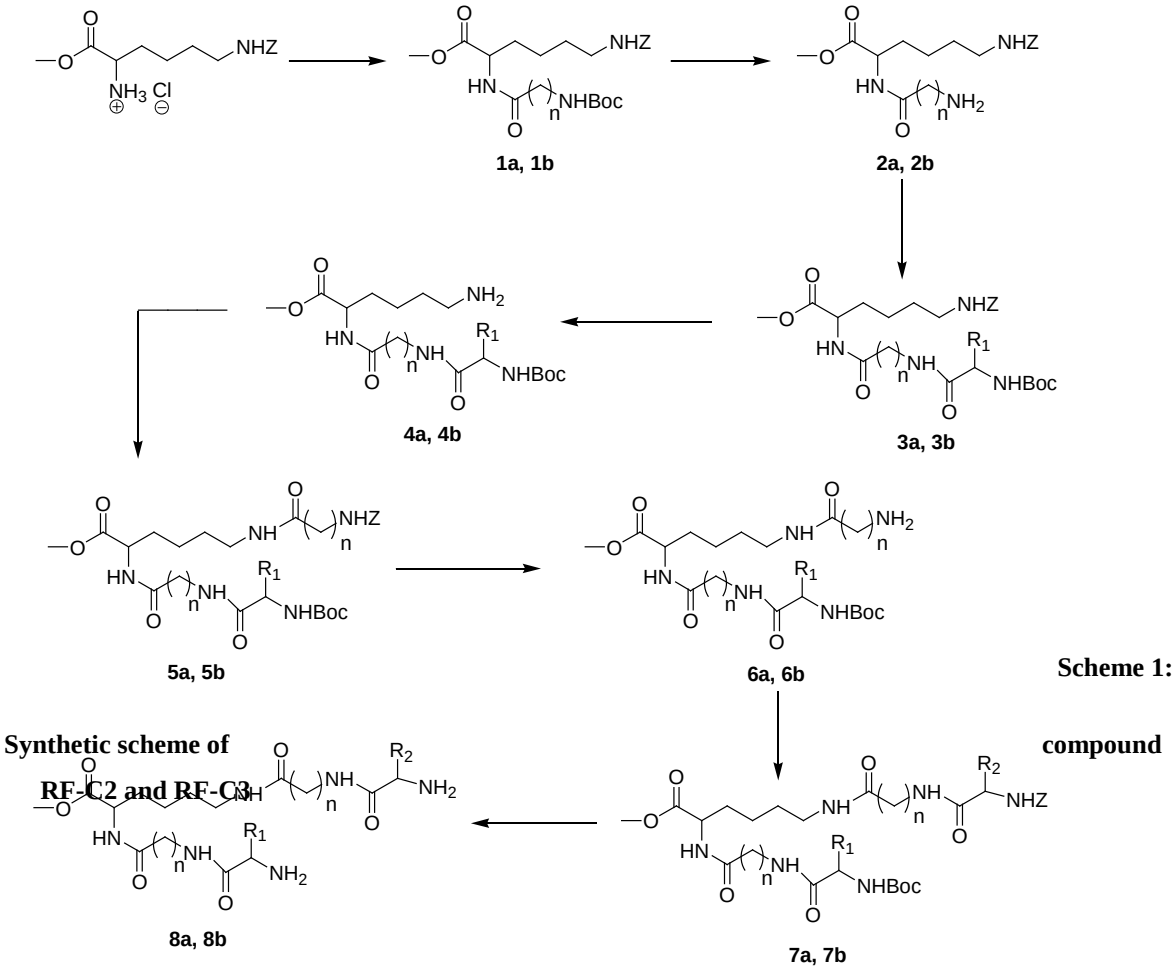
Assessment of cell viability.

Cell viability was assessed using a modified MTT assay.[15] RF-C11 and GV-C11 in DMSO were added at various concentrations (0.1 μM to 1000 μM) to cells. After incubating for 4 h, 25 μL of 5 mg/mL MTT solution was added to the samples and the plates were incubated for 2 h at 37°C. Thiazolyl blue tetrazolium bromide (MTT, Sigma Aldrich) was added to each well (final concentration 0.5 mg/mL) and incubated for 2 h at 37°C in a humidified atmosphere of 95% air and 5% CO_2. HCl (0.08 N) in isopropanol was added to solubilize the blue MTT-formazan product and the cells were incubated for 30 min at room temperature. The absorbance of the solution was read at 570 nm (test) and 630 nm (reference).

ESI Synthesis Schemes and Characterization of RF-Cn Series and Their Derivatives

Overview

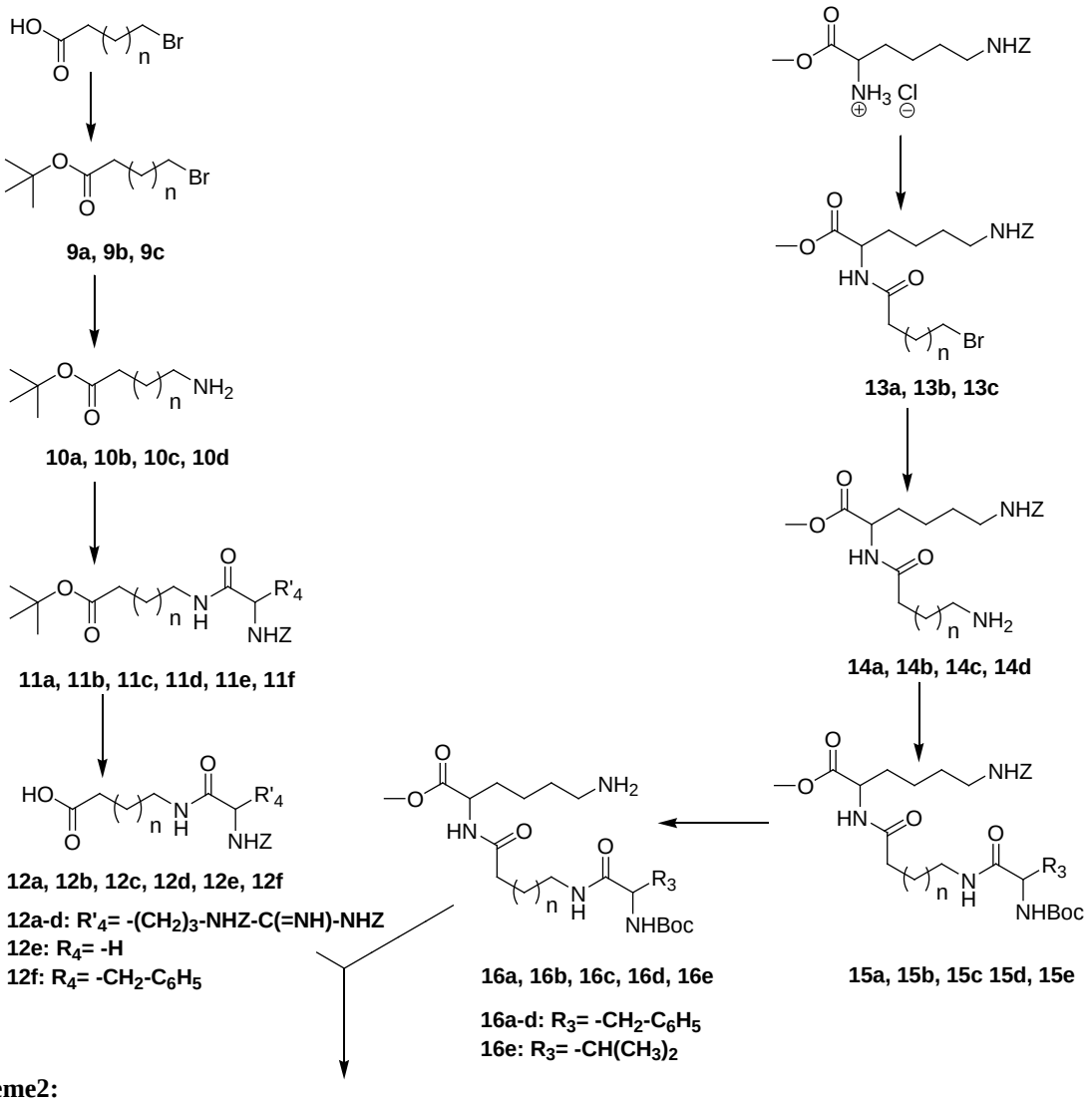
Protected amino acids were purchased from Novabiochem. Organic solvents were purchased from Ranbaxy Fine Chemicals, and other chemicals were from Sigma-Aldrich. ^{1}H NMR spectra were recorded on Varian FT 200 MHz, or Bruker FT 300 MHz. Mass spectra were obtained by Micromass Quattro LC (ESI). Analytical TLC was performed with silica gel-coated aluminum plates (Merck). Purity of final products was determined by using Varian Prostar HPLC with Prostr 325 UV-Vis detector at a flow rate of 1 ml/min in Hibar Purospher



Scheme 1: Synthetic scheme of compound RF-C2 and RF-C3

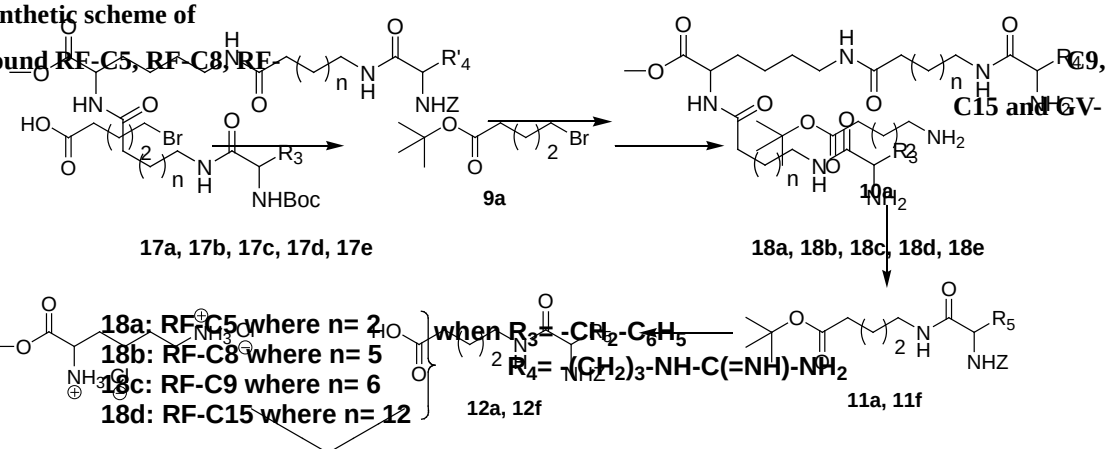
8a: RF-C2 where n= 1
8b: RF-C3 where n= 2

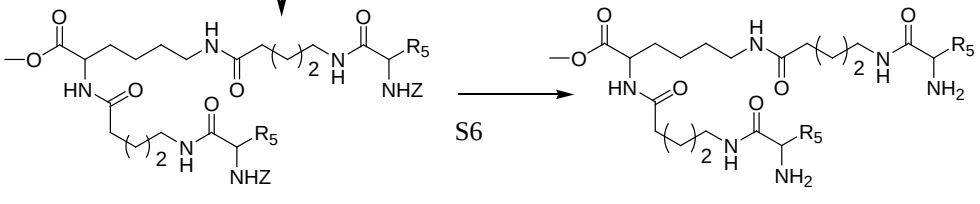
R_1 = $-CH_2-C_6H_5$
R_2 =$-(CH_2)_3-NH-C(=NH)-NH_2$



Scheme2:

Synthetic scheme of compound RF-C5, RF-C8, RF-C9, RF-C15 and GV-C5

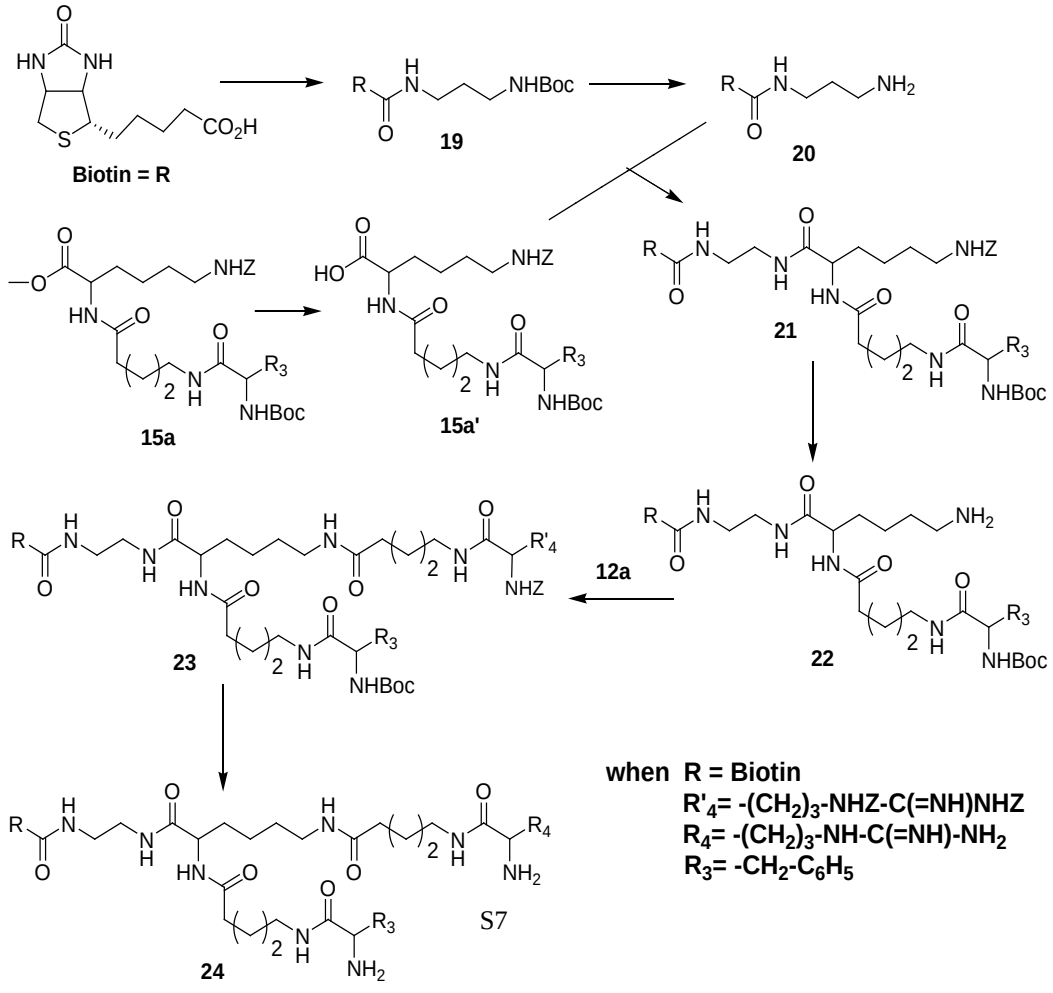




18f: RR-C5 where R_5= -$(CH_2)_3$-NH-C(=NH)-NH_2
18g: FF-C5 where R_5= -CH_2-C_6H_5

Scheme3: Synthetic scheme of compound RR-C5 and FF-C5

STAR column (4.6 mm-250 mm, RP-18e) (Merck). DCM (dichloromethane), DIPEA (diisopropylethylamine), DMF (dimethylformamide), DMSO (dimethyl sulfoxide), EDCI (N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide hydrochloride), HOBt (1-hydroxy-1H-benzotriazole hydrate), r.t. (room temperature), TFA (trifluoroacetic acid), THF (tetrahydrofuran), TMS-OTf (trimethylsilyl trifluoromethanesulfonate).



Scheme4: Synthetic scheme of compound biotinylated-RF-C5 (RF-C5b)

Synthesis of compound RF-C2 and RF-C3

Synthesis of N(ε)-Z-Lysine(-OMe)C2-NHBoc, 1a

HOBt (0.463g, 3.02mmol) and EDCI (0.579g, 3.02mmol) were sequentially added to an ice-cold stirred solution of Boc-Gly-OH (0.529g, 3.02mmol) in dry DCM. After half an hour N(ε)-Z-lysine methyl ester hydrochloride (1g, 3.02mmol) dissolved in dry DCM was added to the reaction mixture. Then DIPEA was added drop wise till the reaction mixture became slightly basic. The resulting solution was stirred at r.t. overnight. Then it was diluted with DCM and washed sequentially with 1N HCl, water and brine. The organic layer was dried and evaporated. The residue was purified by column chromatography using 1% methanol in chloroform. The yield of pure N(ε)-Z-Lysine(-OMe)C2-NHBoc was 92.2% and it was faint yellowish gummy compound. (R_f = 0.55 in 5% methanol in chloroform). **^{1}H NMR** (200MHz, $CDCl_3$): δ = 1.2-1.5 [m, 13H], 1.75-1.9 [m, 2H], 3.1-3.3 [m, 2H], 3.65-3.8 [m, 5H], 4.4-4.6 [q, 1H], 5.05-5.1 [s, 2H], 5.1-5.3 [m, 1H], 5.35-5.5 [m, 1H], 6.9-7.1[d, 1H], 7.2-7.5[s, 5H]. **ESI-MS**: m/z = 452.7 (calculated value for $C_{22}H_{33}O_7N_3$ =451.5).

Synthesis of N(ε)-Z-Lysine(-OMe)C3-NHBoc, 1b

Yield: 86.7% (R_f = 0.55 in 5% methanol in chloroform). **^{1}H NMR** (300MHz, $CDCl_3$): δ = 1.4-1.7 [m, 13H], 1.75-1.9 [m, 2H], 2.3-2.45 [m, 2H], 3.1-3.2 [m, 2H], 3.25-3.4 [m, 2H], 3.7 [s, 3H], 4.45-4.55 [m, 1H], 5.0-5.1 [s, 2H], 5.2-5.3 [m, 1H], 6.5-6.6 [m, 1H], 7.25-7.35[m, 6H]. **ESI-MS**: m/z = 489.0 [$M+Na^+$] (calculated value for $C_{23}H_{35}O_7N_3$ =465.5).

Synthesis of N(ε)-Z-Lysine(-OMe)C2-NH_2, 2a

TFA was added slowly to a solution of N(ε)-Z-Lysine(-OMe)C2-NHBoc (1.2g, 2.66mmol) in dry DCM at 0^0C and the mixture was allowed to stir for 3 hours at r.t. Then DCM and excess TFA were removed by nitrogen flushing. The gummy residue upon chromatographic purification using 2% methanol in chloroform gave 0.84g (90% yield) of pure N(ε)-Z-Lysine(-OMe)C2-NH_2 as a yellowish solid. (R_f = 0.1 in 5% methanol in chloroform). **ESI-MS**: m/z = 352.7 (calculated value for $C_{17}H_{25}O_5N_3$ =351.4).

Synthesis of N(ε)-Z-Lysine(-OMe)C3-NH_2, 2b

Yield: 90% (R_f = 0.1 in 5% methanol in chloroform). **ESI-MS**: m/z = 366 (calculated value for $C_{18}H_{27}O_5N_3$ = 365.4).

Synthesis of N(ε)-Z-Lysine(-OMe)C2F, 3a

HOBt (0.29g, 1.91mmol) and EDCI (0.37g, 1.91mmol) were sequentially added to an ice-cold stirred solution of Boc-Phe-OH (0.51g, 1.91mmol) in dry DCM. After half an hour N(ε)-Z-Lysine(-OMe)C2-NH_2 (0.84g, 2.39mmol) dissolved in dry DCM was added to the reaction mixture. Then DIPEA was added drop wise till the reaction mixture became slightly basic. The resulting solution was stirred at r.t. overnight. Then it was diluted with DCM and washed sequentially with 1N HCl, water and brine. The organic layer was dried and evaporated. The residue upon chromatographic purification using 2% methanol in chloroform gave 1.079g (75.5% yield) of pure N(ε)-Z-Lysine(-OMe)C2F as a faint yellowish liquid. (R_f = 0.5 in 5% methanol in chloroform). **^{1}H NMR** (200MHz, $CDCl_3$): δ = 1.2-1.6 [m, 11H], 1.6-1.9 [m, 4H], 2.8-3.0 [m, 2H], 3.05-3.15 [m, 2H], 3.6 [s, 3H], 3.8-4.0 [m, 1H], 4.15-4.35 [m, 2H], 4.4-4.6 [m, 1H], 5.05 [s, 2H], 5.2-5.3 [m, 1H], 5.4-5.55 [d, 1H], 6.9-7.4 [m, 12H]. **ESI-MS**: m/z = 622 [M+Na^+] (calculated value for $C_{31}H_{42}O_8N_4$ = 598.7).

Synthesis of N(ε)-Z-Lysine(-OMe)C3F, 3b

Yield: 64.9% (R_f = 0.5 in 5% methanol in chloroform). **^{1}H NMR** (300MHz, $CDCl_3$): δ = 1.2-1.6 [m, 15H], 1.6-1.9 [m, 2H], 2.15-2.45 [m, 2H], 2.8-3.25 [m, 4H], 3.7-3.8 [s, 3H], 4.15-4.25 [q, 1H], 4.4-4.5 [q, 1H], 4.9-5.0 [m, 1H], 5.15 [s, 2H], 5.2 [m, 1H], 6.95-7.1 [m, 1H], 7.1-7.4 [m, 11H]. **ESI-MS**: m/z = 635 [M+Na^+] (calculated value for $C_{32}H_{44}O_8N_4$ = 612.7).

Synthesis of Lysine(-OMe)C2F, 4a

N(ε)-Z-Lysine(-OMe)C2F (1g, 1.67mmol) was dissolved in HPLC grade methanol (15ml) . The air of the container was chased by flushing nitrogen several times. 10% Pd on charcoal (0.4g) was added cautiously to the solution and inner walls of the flask were washed down with HPLC grade methanol. Again the air of the flask was chased by nitrogen flushing. Then hydrogen gas was passed through the reaction mixture and it was left stirring for overnight. Then it was filtered through celite and washed with HPLC grade methanol. The combined washings along with the filtrate was evaporated to get 0.816g (90%) of Lysine(-OMe)C2F as a white semi solid. This was used directly in the next reaction without further purification. (R_f = 0.1 in 5% methanol in chloroform). **ESI-MS**: m/z = 465.9 (calculated value for $C_{23}H_{36}O_6N_4$ = 464.6).

Synthesis of Lysine(-OMe)C3F, 4b

Yield: 95.3% (R_f = 0.1 in 5% methanol in chloroform). **ESI-MS**: m/z = 480.0 (calculated value for $C_{24}H_{38}O_6N_4$ = 478.6).

Synthesis of Lysine(-OMe)C2FC2-NHZ, 5a

HOBt (0.183g, 1.2mmol) and EDCI (3.5g, 1.2mmol) were sequentially added to an ice-cold stirred solution of Z-Gly-OH (0.25g, 1.2mmol) in dry DCM. After half an hour Lysine(-OMe)C2F (0.695g, 1.5mmol) dissolved in dry DCM was added to the reaction mixture. Then DIPEA was added drop wise till the reaction mixture became slightly basic. The resulting solution was stirred at r.t. overnight. Then it was diluted with DCM and washed sequentially with 1N HCl, water and brine. The organic layer was dried and evaporated. The residue upon chromatographic purification using 1.5% methanol in chloroform gave 0.69g (70.3% yield) of pure N(ε)-Z-Lysine(-OMe)C2FC2-NHZ as a yellowish semi-solid. (R_f = 0.3 in 5% methanol in chloroform). **^{1}H NMR** (200MHz, $CDCl_3$): δ = 1.2-1.55 [m, 11H], 1.75-1.9 [m, 4H], 2.8-3.5 [m, 4H], 3.6 [m, 5H], 3.6-3.8 [m, 2H], 4.2-4.3 [m, 1H], 4.45-4.6 [m, 1H], 5.1 [s, 2H], 5.65-5.75 [m, 1H], 6.2-6.3 [m, 1H], 6.9-7.0 [m, 1H], 7.1-7.4 [m, 12H]. **ESI-MS**: m/z = 656.9 [$M+H^+$], & 679.1[$M+Na^+$] (calculated value for $C_{33}H_{45}O_9N_5$ = 655.7).

Synthesis of Lysine(-OMe)C3FC3-NHZ, 5b

Yield: 56.8% (R_f = 0.3 in 5% methanol in chloroform). **^{1}H NMR** (200MHz, $CDCl_3$): δ = 1.2-1.9 [m, 15H], 2.25-2.55 [m, 4H], 2.8-3.5 [m, 8H], 3.7 [s, 3H], 4.2-4.3 [m, 1H], 4.35-4.5 [m, 1H], 5.0-5.1 [s, 2H], 5.2-5.35 [m, 1H], 7.1-7.4 [m, 10H]. **ESI-MS**: m/z = 685.3 (calculated

value for $C_{35}H_{49}O_9N_5$ = 683.8).

Synthesis of Lysine(-OMe)C2FC2-NH_2, 6a

N(ε)-Z-Lysine(-OMe)C2FC2-NHZ (0.69g, 1.05mmol) was dissolved in HPLC grade THF (15ml) . The air of the container was chased by flushing nitrogen several times. 10% Pd on charcoal (0.4g) was added cautiously to the solution and inner walls of the flask were washed down with HPLC grade THF. Again the air of the flask was chased by nitrogen flushing. Then hydrogen was passed through the reaction mixture and it was left stirring for overnight. Then it was filtered through celite and washed with HPLC grade THF. The combined washings along with the filtrate was evaporated to afford 0.49g (90%) of Lysine(-OMe)C2FC2-NH_2 as a white semi solid. This was used directly in the next reaction without further purification. (R_f = 0.1 in 5% methanol in chloroform). **ESI-MS**: m/z = 522.8 (calculated value for $C_{25}H_{39}O_7N_5$ = 521.6).

1.4.2.12 Synthesis of Lysine(-OMe)C3FC3-NH_2, 6b

Yield: 90% (R_f = 0.1 in 5% methanol in chloroform). **ESI-MS**: m/z = 551 (calculated value for $C_{27}H_{43}O_7N_5$ = 549.7).

Synthesis of PRF-C2, 7a

HOBt (0.112 g, 0.74mmol) and EDCI (0.141g, 0.74mmol) were sequentially added to an ice-cold stirred solution of Z-Arg-$(Z)_2$-OH (0.424g , 0.74mmol) in dry DCM. After half an hour Lysine(-OMe)C2F2-NH_2 (0.48g, 0.92mmol) dissolved in dry DMF was added to the reaction mixture. Then DIPEA was added dropwise till the reaction mixture became slightly basic. The resulting solution was stirred at r.t. overnight. Then it was diluted with DCM and washed sequentially with 1N HCl, water and brine. The organic layer was dried and evaporated. The residue upon chromatographic purification using 1.5% methanol in chloroform afforded 0.558g (56.1% yield) of pure PRF-C2 as a white gummy semi-solid. (R_f = 0.4 in 5% methanol in chloroform). **^{1}H NMR** (300MHz, $CDCl_3$ + CD_3OD): δ = 1.4-2.2 [m, 19H], 3.0-3.5 [m, 4H], 3.9 [s, 3H], 4.0-4.4 [m, 7H], 4.4-4.7 [m, 2H], 5.2-5.5 [m, 6H], 6.8-7.2 [m, 3H], 7.3-7.6 [m, 20H], 9.9 [s, 1H]. **ESI-MS**: m/z = 1082 [$M+H^+$] & 1104 [$M+Na^+$] (calculated value for $C_{55}H_{69}O_{14}N_9$ = 1080.8).

Synthesis of PRF-C3, 7b

Yield: 64.6% (R_f = 0.4 in 5% methanol in chloroform). **^{1}H NMR** (300MHz, $CDCl_3$): δ = 1.2-1.5 [m, 15H], 1.6-1.8 [m, 4H], 2.3-2.65 [m, 8H], 3.1-3.25 [m, 2H], 3.4-3.6 [m, 4H], 3.7 [s, 3H], 4.05-4.2 [m, 1H], 4.2-4.4 [m, 2H], 5.0-5.4 [m, 6H], 7.15-7.45 [m, 20H].**ESI-MS**: m/z = 1108 (calculated value for $C_{57}H_{73}O_{14}N_9$ = 1108.2).

Synthesis of RF-C2, 8a

PRF-C2 (0.1g, 0.093mmol) was dissolved in TFA (2ml) under nitrogen atmosphere and kept stirred in an ice bath. Thioanisole (0.4ml, 3.4mmol) and TMSOTf (0.6ml, 3.32mmol) were added sequentially to that cooled solution. The reaction mixture was allowed to come to r.t. and stirring was continued for 24 hours. Then reaction was quenched with HPLC grade methanol. Methanol and excess TFA were chased by nitrogen flushing. The residue re-precipitated from methanol-acetone system repeatedly. Finally, upon chloride ion exchange chromatography (using amberlite IRA-400Cl chloride ion exchange resin) gave 0.031g (57% yield) of pure RF-C2 as a yellowish white semi-solid. (R_f = 0.1 in 20% methanol in chloroform). **ESI-HRMS**: m/z = 578.3391 ($[M+H]^+$, calculated value for $C_{26}H_{44}O_6N_9$ = 578.3414). RP-HPLC: Rt = 3.9 min (20% water in acetonitrile), purity >99%.

Synthesis of RF-C3, 8b

Yield: 61.6% (R_f = 0.1 in 20% methanol in chloroform). **ESI-HRMS**: m/z = 606.3728 ($[M+H]^+$, calculated value for $C_{28}H_{48}O_6N_9$ = 606.3727). RP-HPLC: Rt = 2.9 min (20% water in acetonitrile), purity >99%.

Synthesis of compound RF-C5, RF-C8, RF-C9, RF-C15, GV-C5, RR-C5 and FF-C5

Synthesis of *tert*-butyl-5-bromopentanoate, 9a

N, N'-dimethylaminopyridine (1.68g, 13.75mmol) and DCC (6.83g, 33.15mmol) were added sequentially to an ice-cold stirred solution of 5-bromopentanoic acid (5g, 27.62mmol) in dry DCM. After half an hour *tert*-butanol (15.8ml, 165.75mmol) was added to the reaction mixture. The resulting solution was stirred at r.t. overnight. Then it was diluted with DCM and washed sequentially with water and brine. The organic layer was dried and evaporated. The residue upon chromatographic purification using 1% ethyl acetate in hexane afforded 4.1g (62.6% yield) of pure *tert*-butyl-5-bromopentanoate as a light yellow liquid. (R_f = 0.8 in 20% ethyl acetate in hexane). **^{1}H NMR** (200MHz, $CDCl_3$): δ = 1.44 [s, 9H], 1.5-1.6 [m, 2H], 1.84-1.96 [m, 2H], 2.24 [t, 2H], 3.4 [t, 2H]. **ESI-MS**: m/z = 258 (calculated value for $C_9H_{17}O_2Br$ = 237.1).

Synthesis of *tert*-butyl-8-bromooctanoate, 9b

Yield: 70.5% (R_f = 0.85 in 20% ethyl acetate in hexane). **^{1}H NMR** (300MHz, $CDCl_3$): δ = 1.28-1.36 [m, 6H], 1.4-1.48 [s, 9H], 1.55-1.64 [m, 2H], 1.8-1.92 [q, 2H], 2.16-2.24 [t, 2H], 3.36-3.44 [t, 2H].

Synthesis of tert-butyl-15-bromopentadecanoate, 9c

Yield: 75% (R_f = 0.9 in 20% ethyl acetate in hexane). **^{1}H NMR** (300MHz, $CDCl_3$): δ = 1.2-1.36 [m, 20H], 1.36-1.44 [m, 9H], 1.48-1.6 [m, 2H], 1.8-1.92 [q, 2H], 2.12-2.2 [t, 2H], 3.32-3.4 [t, 2H].

Synthesis of *tert*-butyl-5-aminopentanoate, 10a

tert-butyl-5-bromopentanoate (3.6g , 15.19mmol) was dissolved in dry DMF (10ml) and sodium azide (1.48g , 22.77mmol) was added into it. The reaction mixture was heated to 70-80^{0}C and stirred for 17-18 hours. Then it was allowed to cool to r.t. It was diluted with chloroform and sequentially washed with water and brine. The chloroform layer was dried and evaporated. Triphenylphosphine (2.29g, 8.77mmol) was added to the gummy crude material. A vigorous evolution of nitrogen was observed and THF was added to it. When evolution of nitrogen ceased, few drops of water was added to the reaction mixture and stirring was continued for further 4 hours. Then THF was evaporated. The residue upon chromatographic purification using 5% methanol in chloroform afforded 2.79g (96% yield) of pure *tert*-butyl-5-aminopentanoate as a yellow liquid. (R_f = 0.1 in 5% methanol in chloroform). **^{1}H NMR** (200MHz, $CDCl_3$): δ = 1.28 [s, 12 H], 1.44 [s, 9H], 1.5-1.6 [m, 4H], 2.1-2.2 [t, 2H], 2.76-2.84 [t, 2H], 2.92-3.4 [m, 2H].

Synthesis of *tert*-butyl-8-aminooctanoate, 10b

Yield: 90.5% (R_f = 0.1 in 5% methanol in chloroform). **ESI-MS**: m/z = 216.5 (calculated value for $C_{12}H_{25}O_2N$ = 215.3).

Synthesis of *tert*-butyl-9-aminononanoate, 10c

tert-butyl-8-bromooctanoate (1.87g, 6.71mmol) was dissolved in dry DMSO (3ml) and sodium cyanide (2.63g , 53.68mmol) was added. Sodium iodide was added in catalytic amount. The reaction mixture was heated to 80-90^{0}C and stirred for 2-3 hours. Then it was

allowed to cool to r.t. It was diluted with chloroform and sequentially washed with cold water and brine. The organic layer was dried and evaporated. The crude material was dissolved in ice cold dry MeOH and anhydrous nickel chloride (0.081g, 0.625mmol) was added. After some time sodium borohydride (1.65g, 43.54mmol) was added to reaction mixture and left for 2-3 hours. Then reaction mixture was filtered through celite and washed with HPLC grade MeOH. MeOH was driven out and crude was dissolved in chloroform. Work up was done with brine water and dried. The residue upon chromatographic purification using 5% methanol in chloroform afforded 1.26g (88.6% yield) of pure *tert*-butyl-9-aminononanoate as a yellow liquid. (R_f = 0.1 in 5% methanol in chloroform). **ESI-MS**: m/z = 230 (calculated value for $C_{13}H_{27}O_2N$ = 229.4).

Synthesis of *tert*-butyl-15-aminopentadecanoate, 10d

Yield: 85% (R_f = 0.1 in 5% methanol in chloroform).

Synthesis of RC5(-CO_2Bu^t), 11a

HOBt (0.35g, 2.31mmol) and EDCI (0.44g, 2.30mmol) were sequentially added to an ice-cold stirred solution of Z-Arg-$(Z)_2$-OH (1.54g, 2.82mmol) in dry DCM. After half an hour *tert*-butyl-5-aminopentanoate (0.50 g, 2.89mmol) dissolved in dry DCM was added to the reaction mixture. DIPEA was added drop wise to make reaction mixture slightly basic. The resulting solution was stirred at r.t. overnight. Then it was diluted with chloroform and washed sequentially with water and brine. The organic layer was dried and evaporated. The residue upon chromatographic purification using 1% methanol in chloroform afforded 1.73g (72% yield) of pure RC5(-CO_2Bu^t) as a white solid. (R_f = 0.8 in 5% methanol in chloroform). **^{1}H NMR** (200MHz, $CDCl_3$): δ = 1.1-1.2 [m, 2H], 1.4 [s, 9H], 1.5-1.8 [m, 6H], 2.1 [t, 2H], 2.7-2.9 [m, 2H], 3.7-3.8 [m, 2H], 4.2-4.4 [m, 1H], 4.9-5.1 [m, 6H], 6.2 [d, 1H], 6.5-6.6 [t, 1H], 7.2-7.4 [m, 15H], 9.3-9.5 [m, 2H]. **ESI-MS**: m/z = 732 (calculated value for $C_{39}H_{49}O_9N_5$ = 731.8).

Synthesis of RC8(-CO_2Bu^t), 11b

Yield: 65.2% (R_f = 0.85 in 5% methanol in chloroform). **^{1}H NMR** (300MHz, $CDCl_3$+CD_3OD): δ = 1.1-1.35 [m, 10H], 1.45 [s, 9H], 1.5-1.7 [m, 4H], 2.1-2.25 [t, 2H], 2.9-3.1 [t, 2H], 3.8-4.1 [m, 3H, (merged with solvent peak)], 5.05-5.25 [m, 6H], 7.25-7.4 [m, 15H]. **ESI-MS**: m/z = 775 (calculated value for $C_{42}H_{55}O_9N_5$ = 773.9).

Synthesis of RC9(-CO_2Bu^t), 11c

Yield: 60.5% (R_f = 0.85 in 5% methanol in chloroform). **^{1}H NMR** (300MHz, $CDCl_3$): δ = 1.1-1.8 [m, 25H], 2.15-2.25 [m, 4H], 2.3-2.4 [t, 2H], 5.05-5.3 [m, 6H], 7.2-7.4 [m, 15H]. **ESI-MS**: m/z = 789 (calculated value for $C_{43}H_{57}O_9N_5$ = 787.9).

Synthesis of RC15(-CO_2Bu^t), 11d

Yield: 67.5% (R_f = 0.9 in 5% methanol in chloroform).

Synthesis of GC5(-CO_2Bu^t), 11e

Yield: 63.1% (R_f = 0.6 in 5% methanol in chloroform). **^{1}H NMR** (200MHz, $CDCl_3$): δ = 1.4 [s, 9H], 1.5-1.6 [m, 4H], 2.2 [t, 2H], 3.2 [m, 2H], 3.8 [d, 2H], 5.1 [s, 2H], 5.55 [m, 1H], 6.35 [m, 1H], 7.25-7.35 [m, 5H]. **ESI-MS**: m/z = 365 (calculated value for $C_{19}H_{28}O_5N_2$ = 364.4).

Synthesis of FC5(-CO_2Bu^t), 11f

Yield: 58.9% (R_f = 0.7 in 5% methanol in chloroform). **^{1}H NMR** (300MHz, $CDCl_3$): δ = 1.3-1.5 [m, 13H], 2.1-2.2 [t, 2H], 2.9-3.2 [m, 4H], 4.3 [m, 1H], 5.05 [s, 2H], 5.45 [d, 1H], 5.85 [t, 1H], 7.1-7.35 [m, 10H]. **ESI-MS**: m/z = 455 (calculated value for $C_{26}H_{34}O_5N_2$ = 454.6).

Synthesis of RC5(-CO_2H), 12a

TFA was added slowly to a solution of RC5(-CO_2Bu^t) (0.95g, 1.3mmol) in dry DCM at 0^0C and the mixture was allowed to stir for 4 hours at r.t. Then DCM and excess TFA were removed by nitrogen flushing. The gummy residue upon chromatographic purification using 2% methanol in chloroform afforded 0.68g (77.5% yield) of pure RC5(-CO_2H) as a white solid. (R_f = 0.5 in 5% methanol in chloroform). **ESI-MS**: m/z = 676 (calculated value for $C_{35}H_{41}O_9N_5$ = 675.7).

Synthesis of RC8(-CO_2H), 12b

Yield: 81.1% (R_f = 0.5 in 5% methanol in chloroform). **^{1}H NMR** (200MHz, $CDCl_3$+CD_3OD): δ = 1.15-1.4 [m, 10H], 1.5-1.75 [m, 4H], 2.2-2.35 [t, 2H], 2.9-3.1 [m, 2H], 3.35 [m, 2H], 4.1-4.2 [m, 1H], 5.0-5.25 [m, 6H], 7.3-7.5 [m, 15H]. **ESI-MS**: m/z = 718 (calculated value for $C_{38}H_{47}O_9N_5$ = 717.8).

Synthesis of RC9(-CO_2H), 12c

Yield: 75.5% (R_f = 0.5 in 5% methanol in chloroform). **^{1}H NMR** (200MHz, $CDCl_3$+CD_3OD): δ = 1.1-1.4 [m, 12H], 1.5-1.7 [m, 4H], 2.2-2.3 [t, 2H], 2.9-3.0 [t, 2H], 3.8-4.0 [m, 2H], 4.05-4.1 [m 1H] 5.0-5.25 [m, 6H], 7.2-7.4 [m, 15H]. **ESI-MS**: m/z = 732 (calculated value for $C_{39}H_{49}O_9N_5$ = 731.8).

Synthesis of RC15(-CO_2H), 12d

Yield: 79% (R_f = 0.6 in 5% methanol in chloroform).

Synthesis of GC5(-CO_2H), 12e

Yield: 79% (R_f = 0.4 in 5% methanol in chloroform). **ESI-MS**: m/z = 309 (calculated value for $C_{15}H_{20}O_5N_2$ = 308.3).

Synthesis of FC5(-CO_2H), 12f

Yield: 84.4% (R_f = 0.5 in 5% methanol in chloroform). **ESI-MS**: m/z = 399 (calculated value for $C_{22}H_{26}O_5N_2$ = 398.5).

Synthesis of N(ε)-Z-Lysine(-OMe)C5-Br, 13a

HOBt (0.74g, 4.82mmol) and EDCI (1.15g, 6.02mmol) were sequentially added to an ice-cold stirred solution of 5-bromovaleric acid (1.09g, 6.03mmol) in dry DCM. After half an hour N(ε)-Z-lysine methyl ester hydrochloride (2g, 6.04mmol) dissolved in dry DCM was added to the reaction mixture. Then DIPEA was added drop wise till the reaction mixture became slightly basic. The reaction mixture was stirred at r.t. overnight. Then it was diluted with chloroform and washed sequentially with 0.5N HCl, water and brine. The organic layer was dried and evaporated. The residue upon chromatographic purification using 1% methanol in chloroform afforded 1.83g (66% yield) of pure N(ε)-Z-Lysine(-OMe)C5-Br as a white solid. (R_f = 0.6 in 5% methanol in chloroform). **^{1}H NMR** (200MHz, $CDCl_3$): δ = 1.25-1.4 [m, 2H], 1.45-1.6 [m, 2H], 1.65-1.9 [m, 6H], 2.25 [t, 2H], 3.2-3.3 [m, 2H], 3.35 [t, 2H], 3.65 [s, 3H], 4.55 [m, 1H], 4.95 [t, 1H], 5.1 [s, 2H], 6.3 [d, 1H], 7.3 [s, 5H]. **ESI-MS**: m/z = 458 (calculated value for $C_{20}H_{29}O_5N_2Br$ = 457.4).

Synthesis of N(ε)-Z-Lysine(-OMe)C8-Br, 13b

Yield: 64.85% (R_f = 0.7 in 5% methanol in chloroform). **^{1}H NMR** (300MHz, $CDCl_3$): δ = 1.3-1.8 [m, 14H], 1.8-1.9 [m, 2H], 2.15-2.25 [t, 2H], 3.15-3.25 [m, 2H], 3.35-3.4 [t, 2H], 3.75 [s, 3H], 4.55-4.65 [m, 1H], 4.8-4.9 [t, 1H], 5.1 [s, 2H], 6.1 [d, 1H], 7.25-7.4 [s, 5H]. **ESI-MS**: m/z = 501 (calculated value for $C_{23}H_{35}O_5N_2Br$ = 499.4).

Synthesis of N(ε)-Z-Lysine(-OMe)C15-Br, 13c

Yield: 60.1% (R_f = 0.75 in 5% methanol in chloroform). **^{1}H NMR** (300MHz, $CDCl_3$): δ = 1.2-1.7 [m, 26H], 1.7-1.9 [m, 2H], 2.1-2.2 [t, 2H], 3.1-3.2 [q, 2H], 3.3-3.4 [t, 2H], 3.7 [s, 3H], 4.5-4.6 [m, 1H], 4.75-4.85 [m, 1H], 5.05 [s, 2H], 6..0-6.1 [m, 1H], 7.25-7.35 [s, 5H].

Synthesis of N(ε)-Z-Lysine(-OMe)C5-NH_2, 14a

N(ε)-Z-Lysine(-OMe)C5-Br (1.5g , 3.28mmol) was dissolved in dry DMF and sodium azide (0.26g, 3.94mmol) was added. The reaction mixture was heated to 70-80^{0}C and stirred for 17-18 hours. Then it was allowed to cool to r.t. It was diluted with chloroform and sequentially washed with water and brine. The organic layer was dried and evaporated. Triphenylphosphine (1.29g, 4.92mmol) was added to the gummy crude material. A vigorous evolution of nitrogen was observed and THF was added to it. When evolution of nitrogen ceased, few drops of water was added to the reaction mixture and stirring was continued for further 4 hours. Then THF was evaporated in a rotary evaporator. The residue upon chromatographic purification using 5% methanol in chloroform afforded 1.2g (94% yield) of pure N(ε)-Z-Lysine(-OMe)C5-NH_2 as a yellowish gummy liquid compound. (R_f = 0.1 in 5% methanol in chloroform). **ESI-MS**: m/z = 394 (calculated value for $C_{20}H_{31}O_5N_3$ = 393.5).

Synthesis of N(ε)-Z-Lysine(-OMe)C8-NH_2, 14b

Yield: 88.5% (R_f = 0.1 in 5% methanol in chloroform). **ESI-MS**: m/z = 436.8 (calculated value for $C_{23}H_{37}O_5N_3$ = 435.6).

Synthesis of N(ε)-Z-Lysine(-OMe)C9-NH_2, 14c

N(ε)-Z-Lysine(-OMe)C8-Br (1.23g, 2.46mmol) was dissolved in dry DMSO and sodium cyanide (0.97g , 19.72mmol) was added. Sodium iodide was added in catalytic amount. The reaction mixture was heated to 80-90^{0}C and stirred for 2-3 hours. Then it was allowed to cool to r.t. It was diluted with chloroform and sequentially washed with cold water and brine. The organic layer was dried and evaporated. A reddish yellow product was observed. The crude

material was dissolved in ice cold dry MeOH and anhydrous nickel chloride (0.24g, 1.88mmol) was added. After some time sodium borohydride (0.5g, 13.15mmol) was added to reaction mixture and left for 2-3 hours. Then reaction mixture was filtered through celite and washed with HPLC grade MeOH. MeOH was driven out in a rotary evaporator and crude was dissolved in chloroform. Work up was done with brine water and dried. The residue upon chromatographic purification using 5% methanol in chloroform afforded 0.9g (81.8% yield) of pure *tert*-butyl-9-aminononanoate as a yellow liquid. (R_f = 0.1 in 5% methanol in chloroform). **ESI-MS**: m/z = 451 (calculated value for $C_{24}H_{39}O_5N_3$ = 449.6).

Synthesis of N(ε)-Z-Lysine(-OMe)C15-NH$_2$, 14d

Yield: 90.5% (R_f = 0.1 in 5% methanol in chloroform).

Synthesis of N(ε)-Z-Lysine(-OMe)C5F-NHBoc, 15a

HOBt (0.39g, 2.53mmol) and EDCI (0.48g, 2.53mmol) were sequentially added to an ice-cold stirred solution of Boc-Phe-OH (0.54g, 2.04mmol) in dry DCM. After half an hour N(ε)-Z-Lysine(-OMe)C5-NH_2 (1g, 2.54mmol) dissolved in dry DCM was added to the reaction mixture. DIPEA was added to make reaction mixtue slightly basic. The resulting solution was stirred at r.t. overnight. Then it was diluted with chloroform and washed sequentially with water and brine. The organic layer was dried and evaporated. The residue upon chromatographic purification using 2% methanol in chloroform afforded 0.9g (55.28% yield) of pure N(ε)-Z-Lysine(-OMe)C5F as a white gummy semi-solid. (R_f = 0.55 in 5% methanol in chloroform). **^{1}H NMR** (300MHz, $CDCl_3$): δ = 1.3-1.4 [s, 9H], 1.5-1.7 [m, 6H], 2.15-2.35 [t, 2H], 2.9-3.1 [m, 4H], 3.2 [m, 2H], 3.75 [s, 3H], 4.25 [m, 1H], 4.6 [m, 1H], 5.0- [t, 1H], 5.1 [s, 2H], 5.4 [m, 1H], 6.5-6.65 [m, 1H], 7.05 [d, 1H], 7.15-7.4 [m, 10H]. **ESI-MS**: m/z = 641 (calculated value for $C_{34}H_{48}O_8N_4$ = 640.8).

Synthesis of N(ε)-Z-Lysine(-OMe)C8F-NHBoc, 15b

Yield: 61% (R_f = 0.6 in 5% methanol in chloroform). **^{1}H NMR** (200MHz, $CDCl_3$): δ = 1.1-1.8 [m, 25H], 2.1-2.2 [t, 2H], 2.9-3.2[m, 6H], 3.7 [s, 3H], 4.15-4.3 [m, 1H], 4.5-4.6 [m, 1H], 5.0-5.15 [m, 2H], 5.25-5.4 [m, 1H], 6.1-6.2 [m, 1H], 6.45-6.55 [m, 1H], 7.1-7.4 [m, 11H]. **ESI-MS**: m/z = 684 (calculated value for $C_{37}H_{54}O_8N_4$ = 682.9).

Synthesis of N(ε)-Z-Lysine(-OMe)C9F-NHBoc, 15c

Yield: 56.5% (R_f = 0.6 in 5% methanol in chloroform). **^{1}H NMR** (300MHz, $CDCl_3$): δ = 1.1-1.9 [m, 27H], 2.15-2.25 [m, 2H], 2.95-3.3 [m, 6H], 3.65 [s, 3H], 4.2-4.3 [m, 1H], 4.55-4.65 [m, 1H], 5.1 [s, 2H], 5.3-5.4 [m, 1H], 5.9-6.0 [m, 1H], 6.5 [m, 1H], 7.15-7.4 [m, 11H]. **ESI-MS**: m/z = 697 (calculated value for $C_{38}H_{56}O_8N_4$ = 696.9).

Synthesis of N(ε)-Z-Lysine(-OMe)C15F-NHBoc, 15d

Yield: 61.8% (R_f = 0.7 in 5% methanol in chloroform).

Synthesis of N(ε)-Z-Lysine(-OMe)C5V, 15e

Yield: 65% (R_f = 0.6 in 5% methanol in chloroform). **^{1}H NMR** (300MHz, $CDCl_3$): δ = 0.9-1.0 [d, 6H], 1.4 [s, 9H], 1.5-1.9 [m, 10H], 1.95-2.05 [m, 1H], 2.15-2.35 [m, 2H], 3.15-3.2 [m, 4H], 3.4-3.5 [m, 1H] 3.65 [s, 3H], 4.5-4.6 [m, 1H], 5.0 [m, 1H], 5.05 [s, 2H], 5.25 [d, 1H], 6.85 [m, 1H], 7.15 [m,1H], 7.25-7.4 [s, 5H].

Synthesis of Lysine(-OMe)C5F, 16a

N(ε)-Z-Lysine(-OMe)C5F-NHBoc (0.8g, 1.25mmol) was dissolved in HPLC grade methanol. The air of the round bottom flask was chased by flushing nitrogen several times. 10% Pd on charcoal (0.5g) was added cautiously to the solution and inner walls of the flask were washed down with HPLC grade methanol. Again the air of the flask was chased by nitrogen flushing. Then hydrogen was passed through the reaction mixture and it was left stirring for overnight. Then it was filtered through celite and washed with HPLC grade methanol. The combined washings along with the filtrate was evaporated to afford 0.54g (85.1%) of Lysine(-OMe)C5F as a white semi solid. This was used directly in the next reaction without further purification. (R_f = 0.1 in 5% methanol in chloroform). **ESI-MS**: m/z = 507 (calculated value for $C_{26}H_{42}O_6N_4$ = 506.6).

Synthesis of Lysine(-OMe)C8F, 16b

Yield: 80% (R_f = 0.1 in 5% methanol in chloroform). **ESI-MS**: m/z = 549 (calculated value for $C_{29}H_{48}O_6N_4$ = 548.7).

Synthesis of Lysine(-OMe)C9F, 16c

Yield: 88.5% (R_f = 0.1 in 5% methanol in chloroform). **ESI-MS**: m/z = 563 (calculated value for $C_{30}H_{50}O_6N_4$ = 562.7).

Synthesis of Lysine(-OMe)C15F, 16d

Yield: 92.5% (R_f = 0.1 in 5% methanol in chloroform).

Synthesis of Lysine(-OMe)C5V, 16e

Yield: 78.1% (R_f = 0.1 in 5% methanol in chloroform). **ESI-MS**: m/z = 459 (calculated value for $C_{22}H_{42}O_6N_4$ = 458.6).

Synthesis of PRF-C5, 17a

HOBt (0.061 g, 0.33mmol) and HATU (0.15g, 0.39mmol) were sequentially added to an ice-cold stirred solution of RC5(-CO_2H) (0.27g , 0.9mmol) in dry DMF. After half an hour Lysine(-OMe)C5F (0.25g, 0.49mmol) dissolved in dry DMF was added to the reaction mixture. DIPEA was added drop wise to make reaction mixture slightly basic. The resulting solution was stirred at r.t. overnight. Then it was diluted with chloroform and washed sequentially with water and brine. The organic layer was dried and evaporated. The residue upon chromatographic purification using 2% methanol in chloroform afforded 0.33g (57.5% yield) of pure PRF-C5 as a reddish yellow gummy semi-solid. (R_f = 0.5 in 5% methanol in chloroform, v/v). **^{1}H NMR** (300MHz, $CDCl_3$ + CD_3OD): δ = 0.7-0.8 [m, 2H] 1.1-1.2 [s, 9H], 1.2-1.7 [m, 16H], 2.0-2.2 [m, 4H], 2.9-3.1 [m, 4H], 3.3 [t, 4H], 3.6 [s, 3H, (merged with solvent peak)], 3.7 [m, 2H], 3.8-3.9 [m, 1H], 4.05-4.2 [m, 1H], 4.3-4.4 [m, 1H], 4.95-5.25 [m, 6H], 7.15-7.35 [m, 20H]. **ESI-MS**: m/z = 1164 (calculated value for $C_{61}H_{81}O_{14}N_9$ = 1163.4).

Synthesis of PRF-C8, 17b

Yield: 53.6% (R_f = 0.6 in 5% methanol in chloroform). **^{1}H NMR** (200MHz, $CDCl_3$): δ = 1.1-1.4 [m, 33H], 1.4-1.9 [m, 8H], 2.05-2.15 [t, 2H], 2.15-2.25 [t, 2H], 2.75-3.3 [m, 8H], 3.7 [s, 3H], 3.75-3.9 [m, 2H], 3.95-4.1 [m, 1H], 4.25-4.35 [m, 1H], 4.55-5.65 [m, 1H], 4.95-5.3 [m, 6H], 5.7 [m, 1H], 5.95-6.0 [m, 1H], 6.3-6.35 [m, 1H], 6.6 [m, 1H], 7.15-7.45 [m, 20H]. **ESI-MS**: m/z = 1248 (calculated value for $C_{67}H_{93}O_{14}N_9$ = 1248.1).

Synthesis of PRF-C9, 17c

Yield: 61.2% (R_f = 0.6 in 5% methanol in chloroform). **^{1}H NMR** (200MHz, $CDCl_3$ + CD_3OD): δ = 1.2-1.7 [m, 41H], 2.1-2.3 [m, 6H], 3.0-3.2 [m, 4H], 3.3-3.35 [t, 4H], 3.7 [m, 3H], 3.85-4.0 [m, 2H], 5.0-5.3 [m, 6H], 7.15-7.45 [m, 20H]. **ESI-MS**: m/z = 1277 (calculated

value for $C_{69}H_{97}O_{14}N_9$ = 1276.6).

Synthesis of PRF-C15, 17d

Yield: 65.1% (R_f = 0.7 in 5% methanol in chloroform). **^{1}H NMR** (200MHz, $CDCl_3$ + CD_3OD): δ = 1.1-1.4 [m, 20H], 1.5 [s, 9H], 1.5-1.85 [m, 10H], 2.15-2.25 [m, 4H], 2.3-3.0 [m, 4H], 3.75-4.4 [m, 4H], 5.0-5.4 [m, 6H], 6.2-6.3 [m, 2H], 6.5-6.6 [m, 2H], 7.25-7.45 [m, 20H].

Synthesis of PGV-C5, 17e

Yield: 54.5% (R_f = 0.6 in 5% methanol in chloroform). **^{1}H NMR** (300MHz, $CDCl_3$ + CD_3OD): δ = 0.8-1.0 [d, 6H], 1.45-1.55 [s, 9H], 1.55-2.0 [m, 15H], 2.1-2.3 [m, 4H], 3.15-3.3 [m, 6H], 3.65-3.8 [m, 6H], 4.4 [m, (merged with CD_3OD solvent peak), 1H], 5.1 [s, 2H], 7.25-7.4 [m, 5H]. **ESI-MS**: m/z = 749 (calculated value for $C_{37}H_{60}O_{10}N_6$ = 748.9).

Synthesis of PRR-C5, 17f

HATU (0.3g, 0.78mmol) was added to an ice-cold stirred solution of RC5(-CO_2H) (0.53g, 0.78mmol) in dry DMF. After half an hour L-lysine methyl ester di-hydrochloride (0.1g, 0.43mmol) dissolved in dry DMSO was added to the reaction mixture. Then drops of DIPEA were added till the reaction mixture was slightly alkaline. The reaction mixture was stirred at r.t. for 48 hours. Then it was diluted with chloroform and washed sequentially with 0.5N HCl, water and brine. The organic layer was dried and evaporated. The residue upon chromatographic purification using 1% methanol in chloroform afforded 0.38 g (60% yield) of pure PRR-C5 as a white solid. (R_f = 0.7 in 5% methanol in chloroform). **^{1}H NMR** (200MHz, $CDCl_3$ + CD_3OD): δ = 1.3-1.4 [m, 4H], 1.4-1.7 [m, 12H], 2.05-2.25 [m, 4H], 2.9-3.05 [m, 4H], 3.05-3.2 [m, 2H], 3.7 [s, 3H], 3.75-4.0 [m, 6H], 4.35-4.45 [m, 1H], 5.05-5.25 [m, 12H], 7.15-7.4 [m, 30H]. **ESI-MS**: m/z = 1472 (calculated value for $C_{77}H_{94}O_{10}N_6$ = 1447.6).

Synthesis of PFF-C5, 17g

Yield: 59.8% (R_f = 0.6 in 5% methanol in chloroform). **^{1}H NMR** (400MHz, $CDCl_3$ + CD_3OD): δ = 1.3-1.4 [m, 4H], 1.45-1.85 [m, 6H], 2.1-2.3 [m, 4H], 2.75-3.15 [m, 10H], 3.7 [s, 3H], 4.25-4.3 [m, 3H], 4.95-5.1 [m, 4H], 7.15-7.35 [m, 20H]. **ESI-MS**: m/z = 944 [M+Na^+] (calculated value for $C_{51}H_{64}O_{10}N_6$ = 921.1).

Synthesis of RF-C5, 18a

PRF-C5 (0.1g, 0.086mmol) was dissolved in TFA (2ml) under nitrogen atmosphere at 0^0C. Thioanisole (0.4ml, 3.4mmol) and TMS-OTf (0.6ml, 3.32mmol) were added sequentially to that cooled solution. The reaction mixture was allowed to come to r.t. and kept stirring for 24 hours. Then reaction was quenched with HPLC grade methanol. Methanol and excess TFA were chased by nitrogen flushing. The residue was dissolved in minimum amount of HPLC grade methanol and excess diethyl ether was added. A gummy yellowish liquid was precipitated. This was collected by centrifugation and re-precipitated from methanol-diethyl ether system repeatedly. Finally, upon chloride ion exchange chromatography (using amberlite IRA-400Cl chloride ion exchange resin) afforded 0.037g (65% yield) of pure RFC5 as a yellowish semi-solid. (R_f = 0.1in 20% methanol in chloroform). **ESI-HRMS**: m/z = 662.4375 ($[M+H]^+$, calculated value for $C_{32}H_{56}O_6N_9$ = 662.4348). RP-HPLC: Rt = 2.3 min (100% methanol), purity >99%.

Synthesis of RF-C8, 18b

Yield: 68.5% (R_f = 0.1 in 20% methanol in chloroform). **ESI-HRMS**: m/z = 746.5274 ($[M+H]^+$, calculated value for $C_{38}H_{68}O_6N_9$ = 746.5292). RP-HPLC: Rt = 3.8 min (100% methanol), purity >99%.

Synthesis of RF-C9, 18c

Yield: 72% (R_f = 0.1 in 20% methanol in chloroform). **ESI-HRMS**: m/z = 774.5579 ($[M]^+$, calculated value for $C_{40}H_{71}O_6N_9$ = 774.5604). RP-HPLC: Rt = 3.7 min (15% water in acetonitrile), purity >99%.

Synthesis of RF-C15, 18d

Yield: 74.3% (R_f = 0.1 in 20% methanol in chloroform). **ESI**: m/z = 943 ($[M+H]^+$, calculated value for $C_{52}H_{96}O_6N_9$ = 943.3), 472 ($[M+2H]^{2+}$, calculated 472.1). RP-HPLC: Rt = 3.87 min (100% methanol), purity >99%.

Synthesis of GV-C5, 18e

Yield: 60.5% (R_f = 0.1 in 10% methanol in chloroform). **ESI-HRMS**: m/z = 537.33955 ($[M+Na]^+$, calculated value for $C_{24}H_{46}O_6N_6Na$ = 537.3371). RP-HPLC: Rt = 3.8 min (100% methanol), purity >99%.

Synthesis of RR-C5, 18f

Yield: 47% (R_f = 0.0, spot resided at baseline in 20% methanol in chloroform). **ESI-HRMS**: m/z = 336.2387 ($[M+2H]^{2+}$, calculated value for $[C_{29}H_{60}O_6N_{12}]/2$ = 336.2334). RP-HPLC: Rt = 3.6 min (100% methanol), purity >99%.

Synthesis of FF-C5, 18g

Yield: 53% (R_f = 0.1 in 10% methanol in chloroform). **ESI-HRMS**: m/z = 653.4009 ($[M+H]^+$, calculated value for $C_{35}H_{53}O_6N_6$ = 653.4021), 327.2064 ($[M+2H]^{2+}$, calculated 327.2017). RP-HPLC: Rt = 3.9 min (100% methanol), purity >99%.

Synthesis of RF-C11, 18h

RF-C11 (n= 10) was synthesized according to previous work (Lee MJ et al., 2008). Yield: 60.8% (R_f = 0.1 in 20% methanol in chloroform). Final compound was characterized and verified by ESI-MS and purified by HPLC. ESI-MS: m/z = 831 ($[M+H]^+$, calculated value for $C_{44}H_{80}O_6N_9$ = 831.2), 416 ($[M+2H]^+$, calculated value for $C_{44}H_{80}O_6N_9$ = 416.2). RP-HPLC: Rt = 3.78 min (100% methanol), purity >98%.

Synthesis of RF-C5b

Synthesis of Boc-Protected Biotinylated Ethylenediamine, 19

First, 0.19 g HOBt (1.24 mmol), 0.23 g EDCI (1.20 mmol), and 0.20 g biotin (0.82 mmol) was added in 5 ml cold, dry DMF, followed by the addition of 0.20 g DMF solution of mono-BOC-ethylenediamine (1.25 mmol) and DIPEA dropwise. The resulting solution was stirred at r.t. for 16 h. Compound 19 was obtained through column chromatographic purification by using 4% methanol in chloroform (0.21 g 66.4% yield, Rf = 0.40 in 10% methanol in chloroform). ^{1}H NMR (300 MHz, CDCl3 + CD3OD): δ 1.3 [s, 2H], 1.42 [s, 9H], 1.50-1.75 [m, 4H], 2.2-2.4 [t, 2H], 2.7-3.0 [m, 2H], 3.15-3.4 [m, 5H], 4.25-4.4 [m, 2H].

Synthesis of N(ε)Z-lysine(-O-biotinylated ethylenediamine)-C5F, 21

Compound 15a was demethylated by using LiOH in THF:water:methanol (3:1:1) solvent mixture, yielding N(ε)Z-Lysine(OH)-C5F (90% yield, Rf = 0.250 in 5% methanol in chloroform). **ESI-MS**: m/z = 628 [$M+H^+$] (calculated value for $C_{33}H_{47}O_8N_4$ = 627.7).

Compound 19 was subjected to BOC deprotection by using TFA, yielding biotinylated ethylenediamine (Compound **20**, 97.1% yield). Then 0.073 g HOBt (0.48 mmol), 0.091 g EDC (0.48 mmol), and 0.3 g N(ε)Z-Lysine(-OH)-C5F (0.48 mmol) were added in 3 ml cold,

dry DMF, followed by the addition of 0.57 g DMF solution of biotinylated ethylenediamine (0.57 mmol) and dropwise addition of DIPEA. The resulting solution was stirred at r.t. for overnight. After washing with 0.5 N HCl in brine solution and column chromatography by using 4% methanol in chloroform, compound 12 was obtained in pure form (0.256 g, 60%, Rf = 0.50 in 10% methanol in chloroform. ^{1}H NMR (500 MHz, CDCl3 + CD3OD): δ = 1.2-1.8 [m, 25H], 2.15-2.3 [m, 4H], 2.6 [d, 1H], 2.8-2.9 [m, 2H], 3.0-3.2 [m, 5H], 3.35 [m, 2H], 3.5-3.6 [m, 4H], 4.15-4.45 [m, 5H], 5.05 [s, 2H], 7.15-7.4 [m, 10H]. **ESI-MS**: m/z = 918 [M+Na$^+$] (calculated value for $C_{45}H_{66}O_9N_8SNa$ = 918.1).

Synthesis of Lysine(-O-biotinylatedethylenediamine)-C5F, 22

Compound 21 was subjected to *Z*-deprotection by using hydrogenolysis with 10% Pd on charcoal in methanol afforded Lysine(-O-biotinylated ethylenediamine)-C5F (78.8% yield). **ESI-MS**: m/z = 762 [M+H$^+$] (calculated value for $C_{37}H_{60}O_7N_8S$ = 760.9).

Synthesis of PRF-C5b, 23

Then 16 mg HOBt (0.1mmol), 40 mg HATU (0.1mmol), and 71 mg compound 12a (0.1 mmol) was added in 1 ml cold, dry DMF, followed by the addition of 0.13 g DMF solution of Lysine(-O-biotinylated ethylenediamine)-C5F (0.13 mmol) and the dropwise addition of DIPEA. The resulting solution was stirred at r.t. for overnight. Washing with 0.5 N HCl in brine solution and column chromatographic purification by using 5% methanol in chloroform yielded 0.074 g PRF-C5b in pure form (40% yield, Rf = 0.50 in 10% methanol in chloroform). ^{1}H NMR (500 MHz, CDCl3 + CD3OD): δ 1.1 [m, 2H], 1.2-1.7 [m, 31H], 2.05-2.3 [m, 6H], 2.6-2.7 [m, 1H], 3.4 [m, 4H], 3.5-3.6 [m, 1H], 3.8 [m, 1H], 3.9 [m,1H], 4.1-4.2 [m, 4H], 4.25-4.35 [m, 2H], 4.5 [m, 1H], 5.0-5.2 [m, 6H], 7.1-7.4 [m, 20H]. **MALDI-MS**: m/z = 1441 (calculated value for $C_{72}H_{99}O_{15}N_{13}SNa$ = 1441.7)

Synthesis of RF-C5b, 24

PRF-C5b was deprotected and purified through selective precipitation by using methanol/acetone solvent mixture followed by 5% water in acetonitrile yielding pure RF-C5b (30% yield). **ESI-HRMS**: *m/z* 916.55272 ([M+H]$^+$, calculated for $C_{43}H_{74}O_7N_{13}S$ = 916.55494). RP-HPLC: Rt = 2.4 min (20% water in acetonitrile), purity >99%.

HPLC diagram of synthetic inhibitors

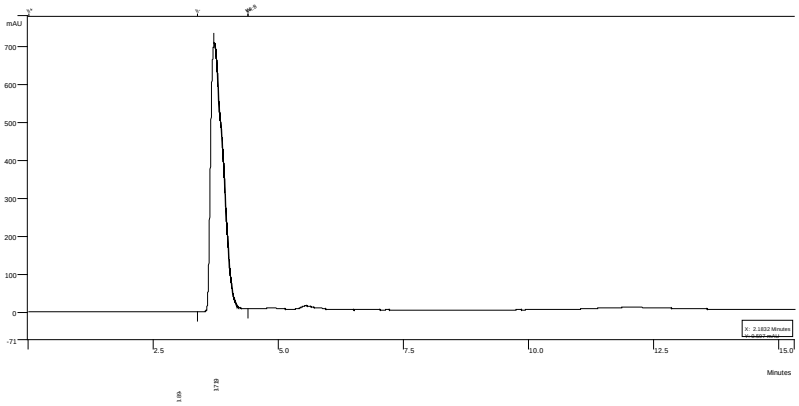


Fig1: HPLC profile of RF-C2 in 20% water in acetonitrile

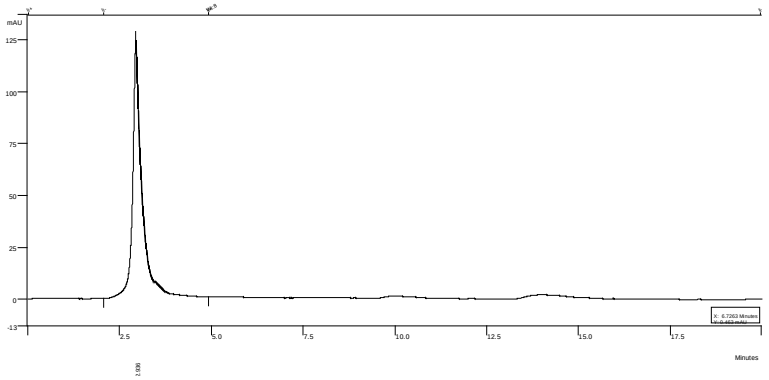


Fig2: HPLC profile of RF-C3 in 20% water in acetonitrile

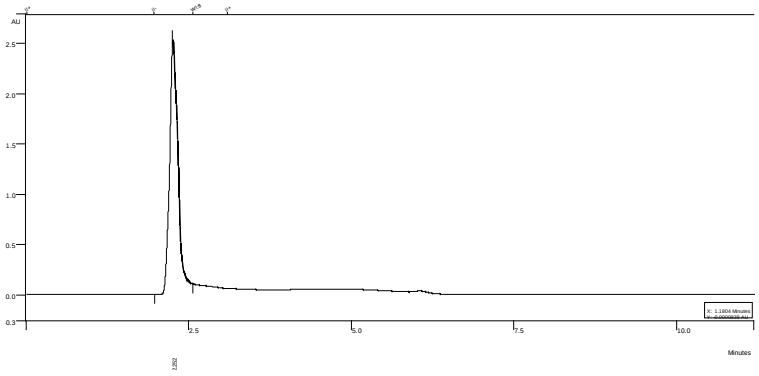


Fig3: HPLC profile of RF-C5 in 100% methanol

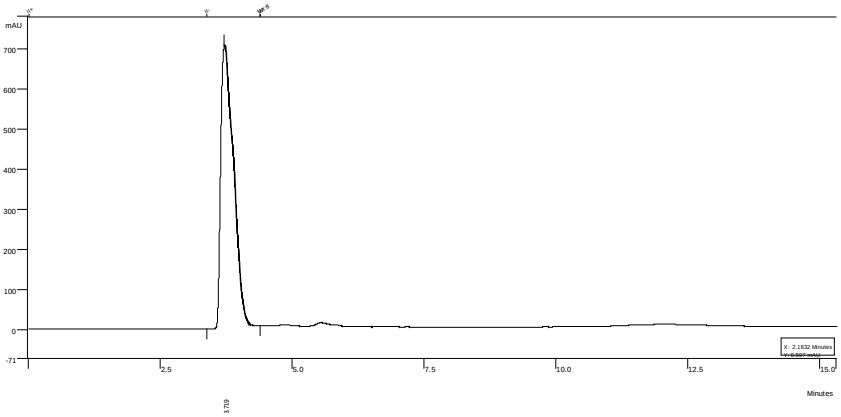


Fig4: HPLC profile of RF-C8 in 100% methanol

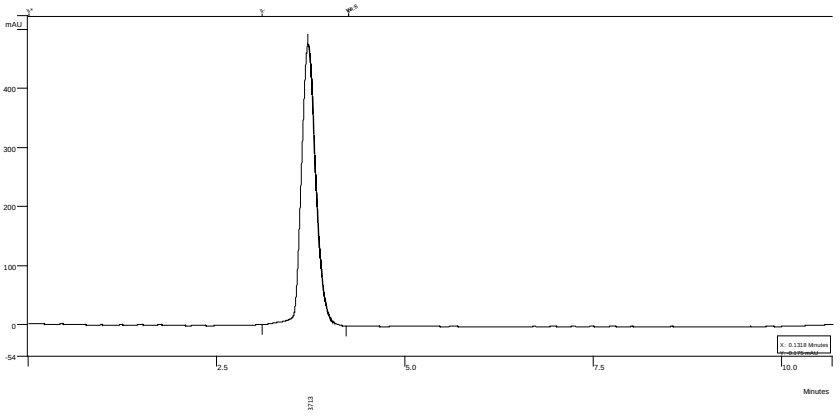


Fig5: HPLC profile of RF-C9 in 15% water in acetonitrile

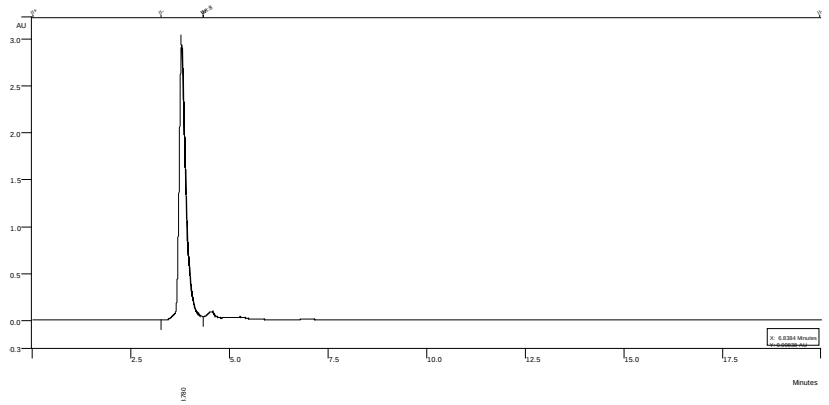


Fig6: HPLC profile of RF-C11 in 100% methanol

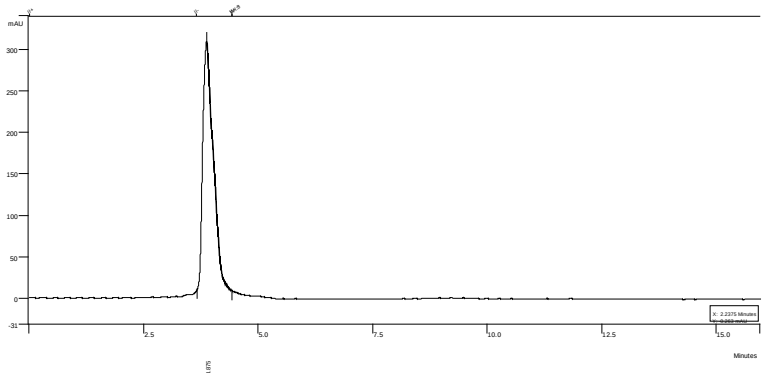


Fig7: HPLC profile of RF-C15 in 100% methanol

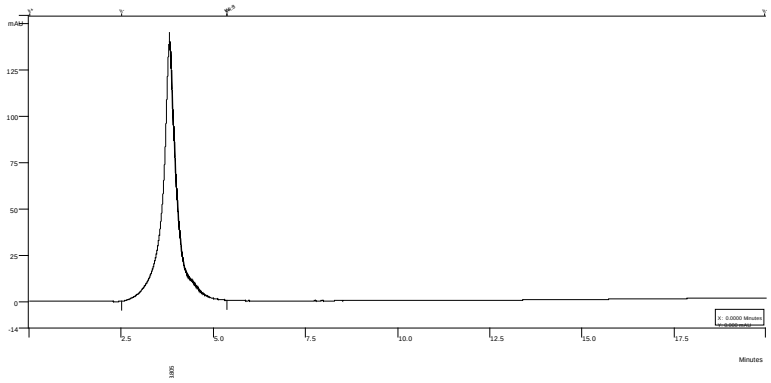


Fig8: HPLC profile of GV-C5 in 100% methanol

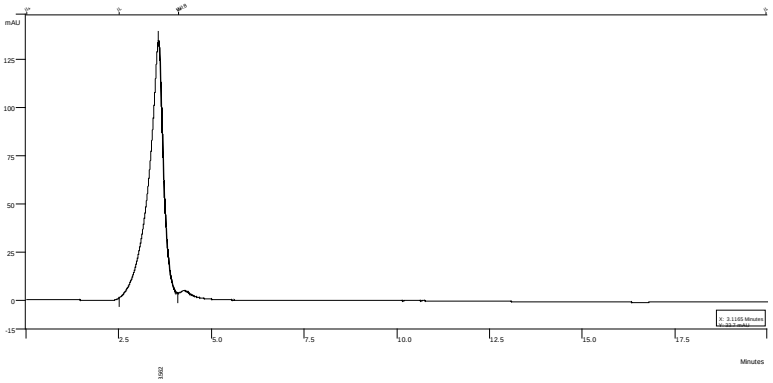


Fig9: HPLC profile of RR-C5 in 100% methanol

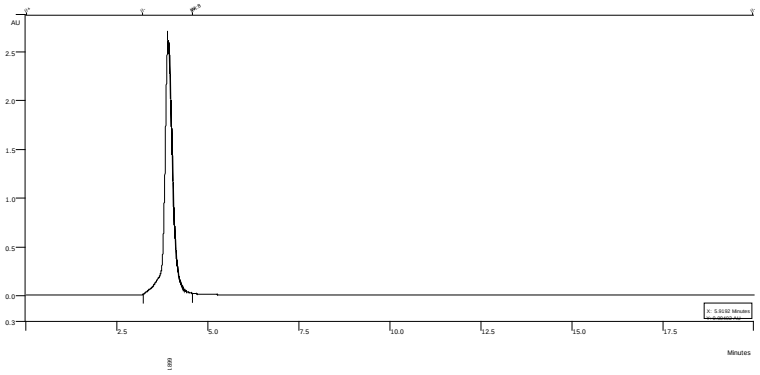


Fig10: HPLC profile of FF-C5 in 100% methanol

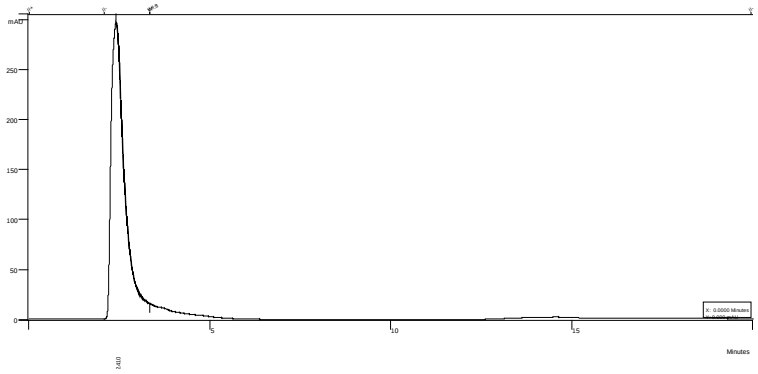


Fig11: HPLC profile of RF-C5b in 20% water in acetonitrile

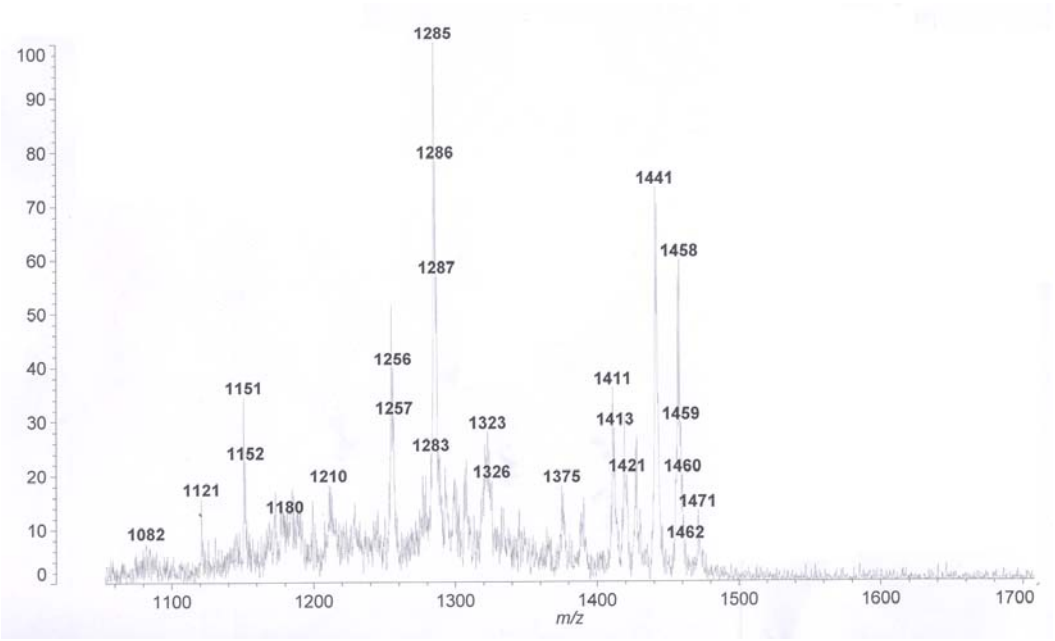
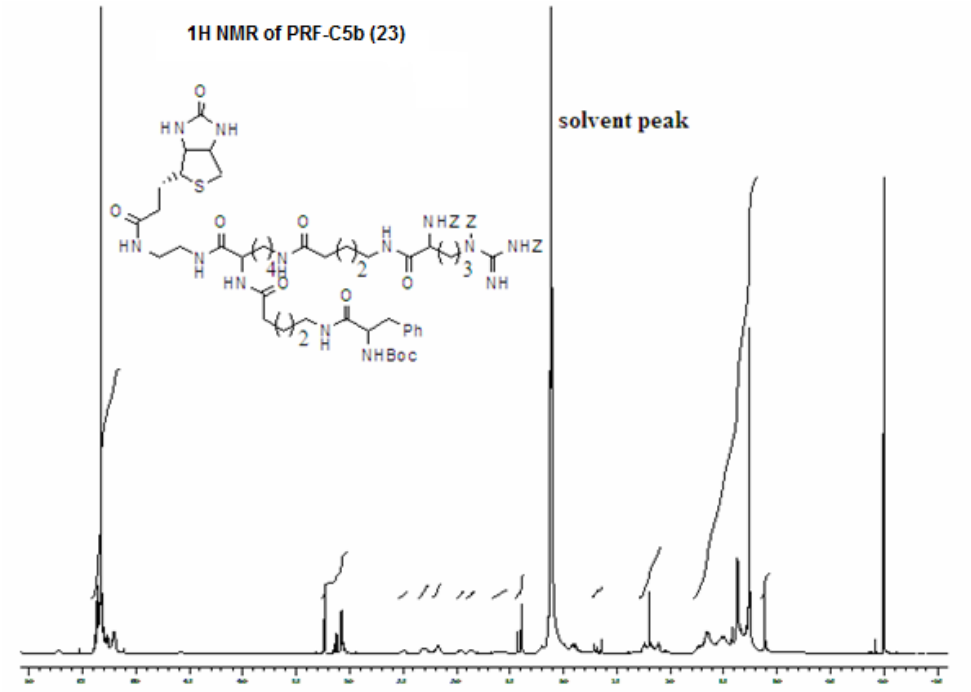
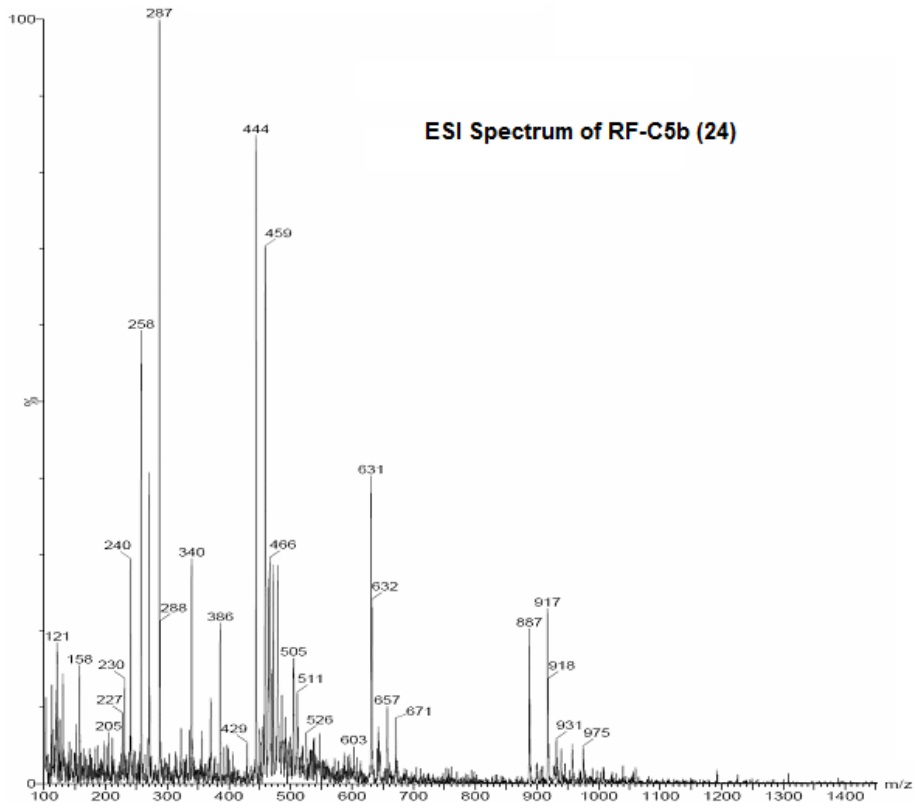
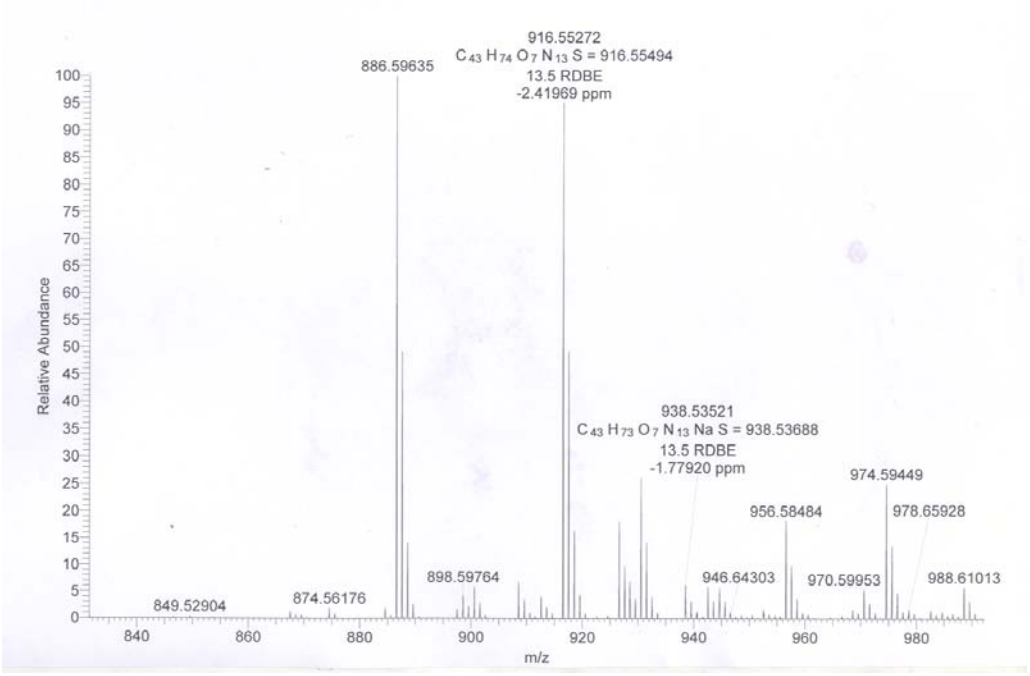


Fig. 12. MALDI-MS of compound 23


1H NMR of PRF-C5b (23)
solvent peak


ESI Spectrum of RF-C5b (24)
287
444
459
258
631
240
340
466
632
917
887
288
386
121
158
230
505
511
918
227
657
671
205
429
526
603
931
975
100
200
300
400
500
600
700
800
900
1000
1100
1200
1300
1400
m/z
%



ESI-HRMS of compound RF-C5b (24)

ESI Reference

1. Lee, M. J., Tasaki, T., Moroi, K., An, J. Y., Kimura, S., Davydov, I. V., and Kwon, Y. T. RGS4 and RGS5 are in vivo substrates of the N-end rule pathway. *Proc. Natl. Acad. Sci. U.S.A.* 2005, **102**, 15030-15035.
2. R. J. De Groot, T. Rumenapf, R. J. Kuhn, E. G. Strauss and J. H. Strauss. Sindbis virus RNA polymerase is degraded by the N-end rule pathway. *Proc. Natl . Acad. Sci. U.S.A.* 1991, **88**, 8967-8971.
3. Y. Shirako and J. H. Strauss. Requirement for an Aromatic Amino Acid or Histidine at the N Terminus of Sindbis Virus RNA Polymerase. *J. Virol.* 1998, **72**, 2310-2315.
4. Kwon, Y. T., Balogh, S. A., Davydov, I. V., Kashina, A. S., Yoon, J. K., Xie, Y., Gaur, A., Hyde, L., Denenberg, V. H., and Varshavsky, A. Altered activity, social behavior, and spatial memory in mice lacking the NTAN1p amidase and the asparagine branch of the N-end rule pathway. *Mol. Cell. Biol.* 2000, **20**, 4135-4148.
5. Kwon, Y. T., Lévy, F., and Varshavsky, A. Bivalent inhibitor of the N-end rule pathway. *J. Biol. Chem.* 1999, **274**, 18135-18139.
6. Tovchigrechko, A., and Vakser, I. A. GRAMM-X public web server for protein–protein docking. *Nucl. Acids Res.* 2006, **34**, W310-W314.
7. Stewart, J. J. Optimization of parameters for semiempirical methods V: modification of NDDO approximations and application to 70 elements. *J. Mol. Model.* 2007, **13**, 1173-1213.
8. Frisch, M. J., Trucks, G. W., Schlegel, H. B., Scuseria, G. E., Robb, M. A., Cheeseman, J. R., Scalmani, G., Barone, V., Mennucci, B., Petersson, G. A., Nakatsuji, H., Caricato, M., Li, X., Hratchian, H. P., Izmaylov, A. F., Bloino, J., Zheng, G., Sonnenberg, J. L., Hada, M., Ehra, M., Toyota, K., Fukuda, R., Hasegawa, J., Ishida, M., Nakajima, T., Honda, Y., Kitao, O., Nakai, H., Vreven, T., Montgomery, Jr., J. A., Peralta, J. E., Ogliaro, F., Bearpark, M., Heyd, J. J., Brothers, E., Kudin, K. N., Staroverov, V. N., Kobayashi, R., Normand, J., Raghavachari, K., Rendell, A., Burant, J. C., Iyengar, S. S., Tomasi, J., Cossi, M., Rega, N., Millam, J. M., Klene, M., Knox, J. E., Cross, J. B., Bakken, V., Adamo, C., Jaramillo, J., Gomperts, R., Stratmann, R. E., Yazyev, O., Austin, A. J., Cammi, R., Pomelli, C., Ochterski, J., Martin, R. L., Morokuma, K., Zakrzewski, V. G., Voth, G. A., Salvador, P., Dannenberg, J. J., Dapprich, S., Daniels, A. D., Farkas, O.,

Foresman, J. B., Ortiz, J. V., Cioslowski, J. and Fox, D. J. GAUSSIAN 09 (Revision A.2), Gaussian, Inc., Wallingford, CT, 2009

9. Sanner, M. F. A component-based software environment for visualizing large macromolecular assemblies. *Structure* 2005, **13**, 447-462.
10. Gasteiger, J., and Marsili, M. Iterative partial equalization of orbital electronegativity—a rapid access to atomic charges. *Tetrahedron* 1980, **36**, 3219-3228.
11. Morris, G. M., Huey, R., Lindstrom, W., Sanner, M. F., Belew, R. K., Goodsell, D. S., and Olson, A. J. AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. *J. Comput. Chem*. 2009, **30**, 2785-2791.
12. Dolinsky, T. J., Czodrowski, P., Li, H., Nielsen, J. E., Jensen, J. H., Klebe, G., and Baker, N. A. PDB2PQR: expanding and upgrading automated preparation of biomolecular structures for molecular simulations. *Nucleic Acids Res*. 2007, **35**, W522-525.
13. Wang, J., Cieplak, P., and Kollman, P. A. How well does a restrained electrostatic potential (RESP) model perform in calculating conformational energies of organic and biological molecules? *J. Comput. Chem.* 2000, **21**, 1049-1074.
14. Baker, N. A., Sept, D., Joseph, S., Holst, M. J., and McCammon, J. A. Electrostatics of nanosystems: application to microtubules and the ribosome. *Proc. Natl. Acad. Sci. U.S.A.* 2001, **98**, 10037-10041.
15. Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* 1983, **65**, 55-63.

ESI Figure and Figure Legends

ESI Fig. S1

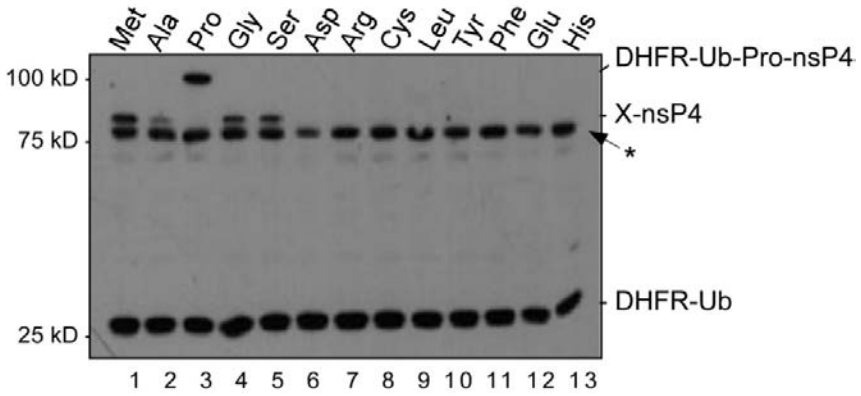


Fig. S1. The steady-state levels of various X-nsP4 model substrates of the N-end rule pathway were examined after 90 min of *in vitro* transcription/translation. Newly synthesized proteins were labeled with biotin-lysyl t-RNA. SDS-PAGE/WB using streptavidin-HRP conjugates. Note that DHFR-Ub-Pro-nsP4 was non-cleavable. *, nonspecific signal. These data complement Fig. 2F.

ESI Fig. S2

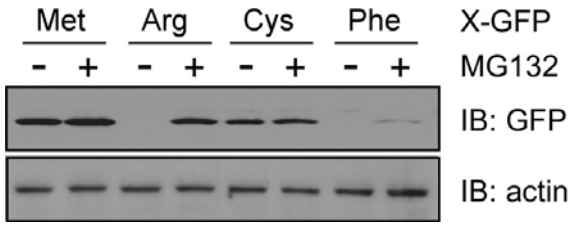


Fig. S2. The proteasome-mediated protein degradation through the N-end rule pathway. Met-, Arg-, Cys-, and Phe-GFP proteins were transiently expressed in HeLa cells. A proteasome inhibitor, MG132 (20 μM), was incubated for 4 hr before cell harvest. Samples were resolved by SDS-PAGE, and anti-GFP antibodies were used for subsequent immunoblotting. actin, loading control.

ESI Fig. S3

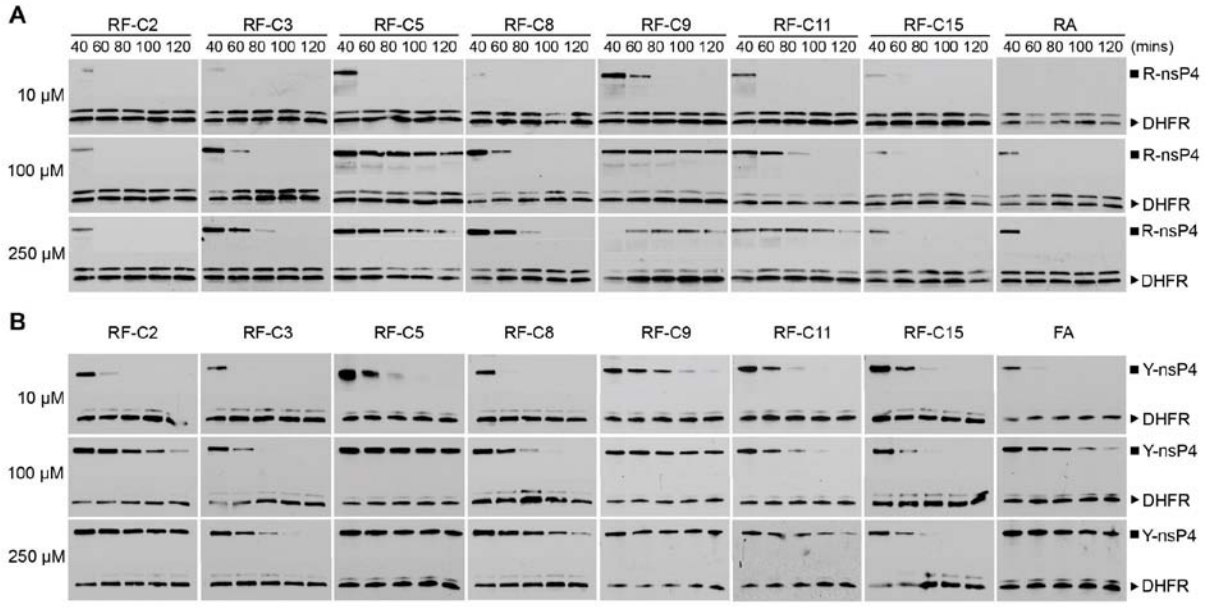


Fig. S3. (A) Heterovalent RF-C5 inhibits the proteolysis of type 1 N-end rule substrate Arg-nsP4 better than dipeptides or heterovalent inhibitors with different tether lengths. The effect of synthesized compounds and dipeptides on Arg-nsP4 degradation was monitored using a time-course Western blot for biotin. Arg-nsP4. DHFR-Ub-Arg-nsP4 fusion proteins were expressed in rabbit reticulocyte lysates. They were cotranslationally cleaved by deubiquitylating enzymes (DUBs) at the Ub-Arg junction yielding a long-lived DHFR-Ub reference and an Arg-nsP4 substrate that is short-lived because Arg is a destabilizing residue of the N-end rule pathway. (B) Same as (A) except using the type 2 model substrate, Tyr-nsP4. Time indicate the reaction time of *in vitro* degradation assays in the presence of heterovalent inhibitors.

ESI Fig. S4

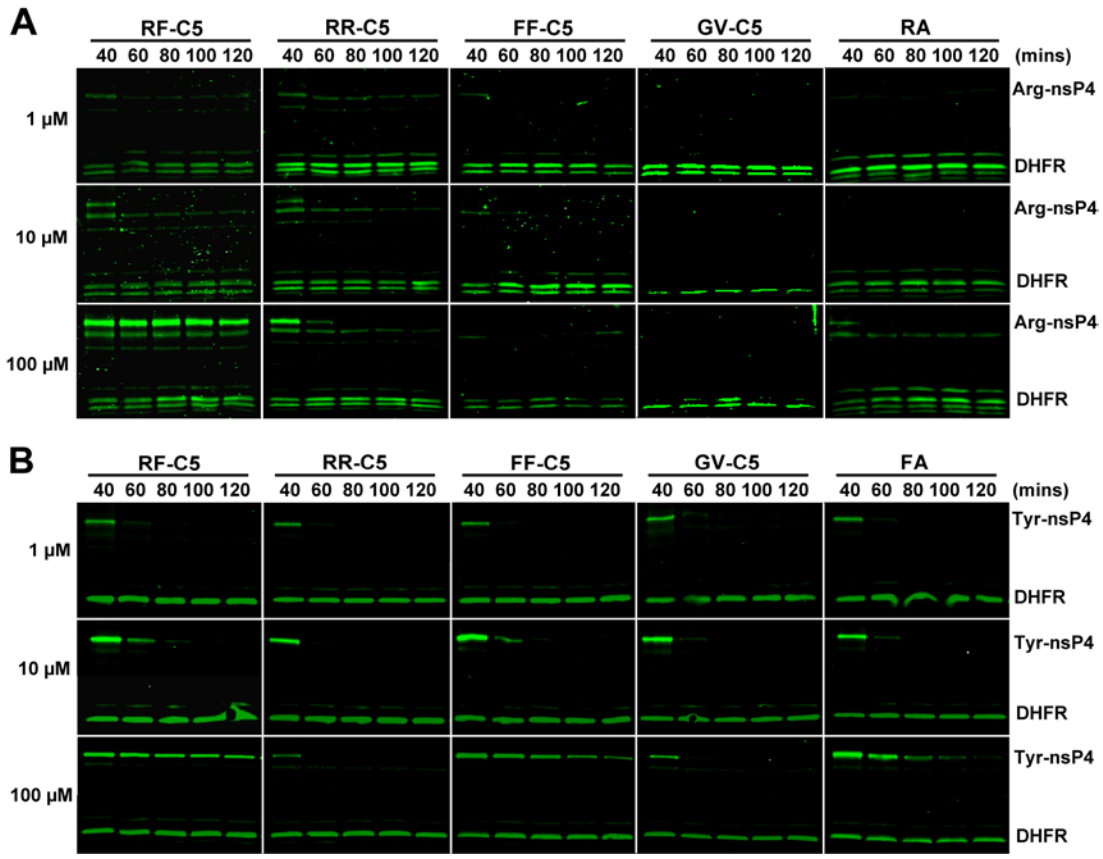


Fig. S4. A and B, same as Supplementary Figs. S3A and S3B, respectively, except the Odyssey system was used instead of a typical WB and various monovalent controls for RF-C5 were used as indicated. Time indicates the total reaction time. These data complement Fig. 4A.

ESI Fig. S5

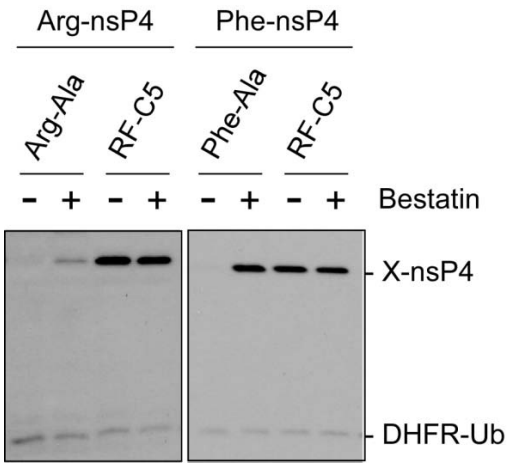


Fig. S5. Heterovalent inhibitors showed higher stability against endopeptidases than dipeptides. The effect of 150 μM bestatin, an endopeptidase inhibitor, on the efficacies of Arg-Ala and Phe-Ala dipeptides were compared with that of RF-C5. Proteins were expressed for 60 min and samples were subject to SDS-PAGE/western blotting using streptavidin-HRP. These data complement Fig. 4D.

ESI Fig. S6

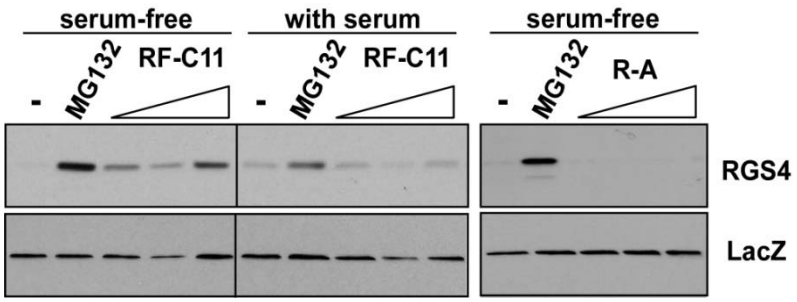


Fig. S6. Heterovalent inhibitors delayed degradation of transiently overexpressed RGS4. Twenty-four hours after cotransfecting MEFs with RGS4 and LacZ plasmids, cells were incubated with 10, 50, or 100 μM RF-C11 or Arg-Ala dipeptides in the presence or absence of serum. RGS4 was strongly stabilized by 10 μM MG132 and more inhibitory effects of heterovalent inhibitors were observed in the serum-free condition.

ESI Fig. S7

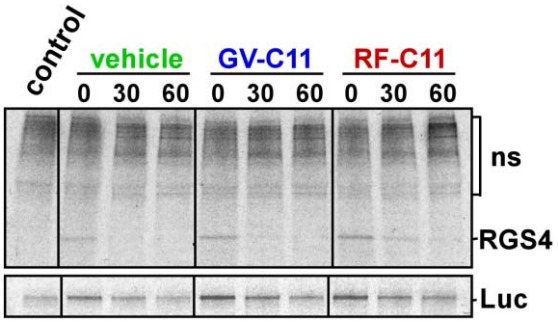


Fig. S7. Pulse-chase analysis of RGS4, a physiological N-end rule substrate, using heterovalent inhibitors showed delayed degradation of newly synthesized RGS4 protein in MEFs. Transfected cells were preincubated with 100 μM RF-C11 or GV-C11, labeled for 12 min with ^{35}S-Met/Cys, followed by anti-RGS4 immunoprecipitation, SDS-PAGE analysis, and autoradiography.