Supporting Information for

Design and structure-activity relationship of a potent furin inhibitor derived from influenza hemagglutinin

Monika A. Lewandowska-Goch,[†,⊥] Anna Kwiatkowska,[*,‡,§,⊥] Teresa Łepek,[†] Kevin Ly,[#] Pauline Navals,[‡,∥,§] Hugo Gagnon,[#] Yves L. Dory,[‡,∥] Adam Prahl[†] and Robert Day[*,‡,§]

[†] Department of Organic Chemistry, Faculty of Chemistry, University of Gdańsk, Wita Stwosza 63, 80-308 Gdańsk, Poland

[‡] Institut de pharmacologie de Sherbrooke, Université de Sherbrooke, 3001 12e Avenue Nord, Sherbrooke J1H 5N4, Canada

[§] Département de Chirurgie/Urologie, Faculté de Médecine et Sciences de la Santé, Centre Hospitalier Universitaire de Sherbrooke, 3001 12e Avenue Nord, Sherbrooke J1H 5N4, Canada

[#] PhenoSwitch Bioscience Inc., 975 rue Léon-Trépanier, Sherbrooke QC J1G 5J6, Canada

[∥] Département de chimie, Faculté des Sciences, Université de Sherbrooke, 3001 12e Avenue Nord, Sherbrooke J1H 5N4, Canada

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Material and methods

Reagents

All amino acids derivatives, coupling reagents and solvents were obtained from Bachem, Iris Biotech, GL Biochem, Novabiochem, Merck, POCH, Fluka, Sigma-Alderich. Tenta Gel S RAM resin was purchased from Rapp Polymere (Tübingen, Germany).

Peptide Synthesis

The peptides were synthesized according to standard Fmoc/tBu strategy and standard coupling procedures[1]. The synthesis was conducted using a peptide synthesizer Symphony (Protein Technologies). All analogs with Arg at the P1 position were obtained on a Tenta Gel S RAM polystyrene resin (Rapp Polymere, capacity 0.24 mmol/g) using a 3-fold excess of the protected amino acids and following coupling agents: 2-(7-aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU, 4 equiv), 1-hydroxy-7-azabenzotriazole (HOAt, 4 equiv) in the presence of 4-methylmorpholine (NMM, 8 equiv) in DMF. The Fmoc groups were removed by 20% piperidine solution in DMF. The last step of the synthesis was *N*-terminal acetylation which was carried out in a mixture of acetic anhydride/*N,N*-diisopropylamine (DIPEA)/dichloromethane (DCM), (15:15:70 v/v/v) in 20 minutes. After the completion of the synthesis, the product was cleaved from the resin. It was accomplished by the cocktail of trifluoracetic acid (TFA)/water/ triisopropylosilane (TIS) (95:2.5:2.5 v/v/v) in 3 h at the room temperature. The solutions of the released peptides were filtered, washed three times by the cleavage cocktail and concentrated in *vacuo*. The obtained compounds were precipitated in cold diethyl ether and centrifuged. The obtained precipitate was filtered, dissolved in water and lyophilized.

One cyclic analog was designed and synthesized. This peptide was obtained using 2-chlorotrityl chloride resin in order to obtain a free carboxylic acid group at the *C*-terminus, which was further cyclized with the ε-amino group of the Lys side chain (protected during the synthesis with *N*-methyltrityl (Mtt) moiety) at the P5 position. Cleavage of the compound with the simultaneous deprotection of Mtt was conducted in mild acidic conditions using the mixture of hexafluoro-2-propanol (HFIP)/DCM (1:4, v:v). Cyclization through the formation of amide bond was performed using benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP, 3 equiv), 1-hydroxy-6-chloro-benzotriazole (6Cl-HOBt, 3 equiv) and DIPEA (9 equiv) in anhydrous DMF. After completion of reaction (monitored by HPLC), the solvents were evaporated, and the remaining protecting groups were removed using a mixture of trifluoracetic acid (TFA)/water/ triisopropylosilane (TIS) (95:2.5:2.5 v/v/v). The obtained analog was lyophilized and purified.

The all crude peptides were purified by semipreparative reverse phase HPLC using a C_{18} column (Jupiter C_{18}, 5 µm, 250x10,00 mm, 300 Å, Phenomenex) in two steps. Two solvent systems were used during the purification to obtain desired compounds. First, peptides were purified using: [A] 0,1% aq heptafluorobutyric acid (HFBA) and [B] 80% acetonitrile (ACN) in [A], which allows facile elimination of hydrophilic impurities by increasing their affinity to reverse phase HPLC columns[2]. After determination of the presence of desired peptides, analogs were purified using a traditional solvent system: [A] 0,1% aq TFA and [B] 80% ACN in [A]. The purity of the final peptides was confirmed by analytical HPLC and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The purity of the peptides exceeded 95%.

Enzyme kinetic studies

$K_{i's}$ were determined via competitive kinetic assays with soluble recombinant human furin (*h*furin) and for the selected peptides with soluble recombinant human PACE4, PC5/6 and PC7. All measurements were performed on a Gemini EM 96-well spectrofluorometer (Molecular Devices; Sunnyvale, CA, USA) (λ_{em}, 370 nm; λ_{ex}, 460 nm; cutoff, 435 nm) at fixed concentration of substrate (pyroGlu-Arg-Thr-Lys-Arg-methyl-coumaryl-7-amide, Bachem, Switzerland) and enzyme (for details see Table S3) at 37 °C over a period of 1 h. Kinetics assays were analyzed using SoftMaxPro5, and K_i values were determined from IC_{50} using Cheng and Prusoff's equation[3].

Stability studies

5 μl of an aqueous peptide solution was added to 25 μl of mouse plasma (CD1 mice, from mixed-sex animals collected with heparin sodium; Innovative Research; Novi, MI, USA) to reach a final concentration of 500 ng/mL. the prepared samples were incubated at the selected time points at 37°C. Each time point was done in triplicate. The reaction was quenched by the addition of 60 µL of 2% aqueous formic acid (FA) containing an internal standard (IS), i.e. CF63. The obtained samples were diluted in 0.1% aqueous TFA and cleaned using the Strata-X polymeric reversed phase solid phase extraction (SPE) 96-well plates (10mg/well) from Phenomenex (Torrance, CA, USA). Eluted peptides were dried overnight under nitrogen stream and re-suspended in 30 uL of 0.1% aqueous FA. Acquisition was performed with a Shimadzu LCMS-8060 (Shimadzu, Kyoto, Japan) equipped with an electrospray interface with a 100 µm iD capillary and coupled to a Nexera XR HPLC (Shimadzu, Kyoto, Japan). LabSolution v5.93 software was used to control the instrument and for data processing and acquisition. Optimized Multiple Reaction Monitoring (MRM) parameters were used to monitor each peptide. Samples were injected (5 µL) and analyzed by LC-MS/MS. Separation was performed on a Luna Omega Polar from Phenomenex 100mmx2.1mm (3um particles) which was maintained at 50°C. For the 5.5 min LC gradient, the mobile phase consisted of the following solvent A (0.1% aqueous FA) and solvent B (0.1% FA in ACN) at a flow rate of 500 µL/min. Gradient started at 100:0; A:B. Data integration and quantification was performed with LabSolutions (Shimadzu) using the area under the curve. The values were normalized to IS and 0 h time point to determine $t_{1/2}$ + SEM using GraphPad Prism one-phase decay curve-fit.

Table S1. Analytical data of the peptides used in the present study.

a) Analogs from the P5, P6, P7 and P8 series.

Code	Modified position	Peptide sequence	Retention time[a] t_R [min]	Molecular mass	
				Calculated	Found[b]
CF1	**-**	*Ac*-RARRRKKRT-NH_2	13.089	1268.8	1269.0
CF2	**Ala^{P5}**	*Ac*-RAR**A**RKKRT-NH_2	13.031	1183.7	1183.7
CF3	**Asp ^{P5}**	*Ac*-RAR**D**RKKRT-NH_2	12.445	1227.7	1227.9
CF4	**Glu ^{P5}**	*Ac*-RAR**E**RKKRT-NH_2	13.226	1241.7	1241.8
CF5	**Phe ^{P5}**	*Ac*-RAR**F**RKKRT-NH_2	16.894	1259.8	1259.9
CF6	**Gly ^{P5}**	*Ac*-RAR**G**RKKRT-NH_2	12.984	1169.7	1169.7
CF7	**His ^{P5}**	*Ac*-RAR**H**RKKRT-NH_2	13.226	1249.8	1249.9
CF8	**Ile ^{P5}**	*Ac*-RAR**I**RKKRT-NH_2	16.767	1225.8	1225.8
CF9	**Lys ^{P5}**	*Ac*-RAR**K**RKKRT-NH_2	12.815	1240.8	1240.9
CF10	**Leu ^{P5}**	*Ac*-RAR**L**RKKRT-NH_2	16.593	1225.8	1226.0
CF11	**Met ^{P5}**	*Ac*-RAR**M**RKKRT-NH_2	15.585	1243.7	1243.8
CF12	**Asn ^{P5}**	*Ac*-RAR**N**RKKRT-NH_2	12.654	1226.7	1226.9
CF13	**Pro ^{P5}**	*Ac*-RAR**P**RKKRT-NH_2	13.433	1209.8	1209.7
CF14	**Gln ^{P5}**	*Ac*-RAR**Q**RKKRT-NH_2	12.701	1240.8	1240.9
CF15	**Ser ^{P5}**	*Ac*-RAR**S**RKKRT-NH_2	12.495	1199.8	1299.5
CF16	**Thr ^{P5}**	*Ac*-RAR**T**RKKRT-NH_2	13.437	1213.8	1213.5
CF17	**Val ^{P5}**	*Ac*-RAR**V**RKKRT-NH_2	14.989	1211.8	1211.7
CF18	**Trp ^{P5}**	*Ac*-RAR**W**RKKRT-NH_2	17.676	1298.8	1298.8
CF19	**Tyr ^{P5}**	*Ac*-RAR**Y**RKKRT-NH_2	14.863	1275.8	1275.8
CF20	**Ala ^{P6}**	*Ac*-RA**A**RRKKRT-NH_2	12.823	1183.7	1184.0
CF21	**Asp ^{P6}**	*Ac*-RA**D**RRKKRT-NH_2	12.638	1227.7	1228.1
CF22	**Glu ^{P6}**	*Ac*-RA**E**RRKKRT-NH_2	13.832	1241.7	1242.0
CF23	**Phe ^{P6}**	*Ac*-RA**F**RRKKRT-NH_2	18.726	1259.8	1260.1
CF24	**Gly ^{P6}**	*Ac*-RA**G**RRKKRT-NH_2	12.372	1169.7	1169.6
CF25	**His ^{P6}**	*Ac*-RA**H**RRKKRT-NH_2	12.560	1249.8	1249.8
CF26	**Ile ^{P6}**	*Ac*-RA**I**RRKKRT-NH_2	15.746	1225.8	1225.9
CF27	**Lys ^{P6}**	*Ac*-RA**K**RRKKRT-NH_2	12.750	1240.8	1240.4
CF28	**Leu ^{P6}**	*Ac*-RA**L**RRKKRT-NH_2	17.141	1225.8	1225.2
CF29	**Met ^{P6}**	*Ac*-RA**M**RRKKRT-NH_2	14.233	1243.7	1243.6
CF30	**Asn ^{P6}**	*Ac*-RA**N**RRKKRT-NH_2	12.449	1226.7	1226.6
CF31	**Pro ^{P6}**	*Ac*-RA**P**RRKKRT-NH_2	13.699	1209.8	1209.4
CF32	**Gln ^{P6}**	*Ac*-RA**Q**RRKKRT-NH_2	11.587	1240.8	1241.0
CF33	**Ser ^{P6}**	*Ac*-RA**S**RRKKRT-NH_2	12.888	1199.8	1199.9
CF34	**Thr ^{P6}**	*Ac*-RA**T**RRKKRT-NH_2	12.836	1213.8	1213.9
CF35	**Val ^{P6}**	*Ac*-RA**V**RRKKRT-NH_2	15.309	1211.8	1211.9
CF36	**Trp ^{P6}**	*Ac*-RA**W**RRKKRT-NH_2	19.038	1298.8	1298.8
CF37	**Tyr ^{P6}**	*Ac*-RA**Y**RRKKRT-NH_2	15.310	1275.8	1275.6
CF38	**Asp ^{P7}**	*Ac*-R**D**RRRKKRT-NH_2	12.565	1312.8	1312.8
CF39	**Glu ^{P7}**	*Ac*-R**E**RRRKKRT-NH_2	10.361	1326.8	1327.0
CF40	**Phe ^{P7}**	*Ac*-R**F**RRRKKRT-NH_2	17.746	1344.8	1344.7
CF41	**Gly ^{P7}**	*Ac*-R**G**RRRKKRT-NH_2	12.458	1254.8	1254.6
CF42	**His ^{P7}**	*Ac*-R**H**RRRKKRT-NH_2	13.499	1334.8	1334.7
CF43	**Ile ^{P7}**	*Ac*-R**I**RRRKKRT-NH_2	16.403	1310.8	1311.1
CF44	**Lys ^{P7}**	*Ac*-R**K**RRRKKRT-NH_2	12.910	1325.9	1325.8

CF45	**Leu** ^{P7}	*Ac*-R**L**RRRKKRT-NH_2	17.179	1310.8	1310.9
CF46	**Met** ^{P7}	*Ac*-R**M**RRRKKRT-NH_2	15.207	1328.8	1329.0
CF47	**Asn** ^{P7}	*Ac*-R**N**RRRKKRT-NH_2	12.241	1311.8	1311.8
CF48	**Pro** ^{P7}	*Ac*-R**P**RRRKKRT-NH_2	13.710	1294.8	1294.9
CF49	**Gln** ^{P7}	*Ac*-R**Q**RRRKKRT-NH_2	13.320	1325.8	1326.1
CF50	**Arg** ^{P7}	*Ac*-R**R**RRRKKRT-NH_2	15.217	1353.9	1353.9
CF51	**Ser** ^{P7}	*Ac*-R**S**RRRKKRT-NH_2	9.939	1284.8	1285.0
CF52	**Thr** ^{P7}	*Ac*-R**T**RRRKKRT-NH_2	10.449	1298.8	1299.1
CF53	**Val** ^{P7}	*Ac*-R**V**RRRKKRT-NH_2	14.461	1296.8	1296.8
CF54	**Trp** ^{P7}	*Ac*-R**W**RRRKKRT-NH_2	17.974	1383.8	1383.8
CF55	**Tyr** ^{P7}	*Ac*-R**Y**RRRKKRT-NH_2	15.610	1360.8	1361.0
CF56	**Ala** ^{P8}	*Ac*-**A**ARRRKKRT-NH_2	12.841	1183.7	1183.9
CF57	**Asp** ^{P8}	*Ac*-**D**ARRRKKRT-NH_2	12.278	1227.7	1228.1
CF58	**Glu** ^{P8}	*Ac*-**E**ARRRKKRT-NH_2	12.528	1241.7	1242.2
CF59	**Phe** ^{P8}	*Ac*-**F**ARRRKKRT-NH_2	18.975	1259.8	1260.2
CF60	**Gly** ^{P8}	*Ac*-**G**ARRRKKRT-NH_2	12.042	1169.7	1170.4
CF61	**His** ^{P8}	*Ac*-**H**ARRRKKRT-NH_2	12.441	1249.8	1250.2
CF62	**Ile** ^{P8}	*Ac*-**I**ARRRKKRT-NH_2	17.277	1225.8	1226.3
CF63	**Lys** ^{P8}	*Ac*-**K**ARRRKKRT-NH_2	13.524	1240.8	1241.3
CF64	**Leu** ^{P8}	*Ac*-**L**ARRRKKRT-NH_2	18.004	1225.8	1226.0
CF65	**Met** ^{P8}	*Ac*-**M**ARRRKKRT-NH_2	15.702	1243.7	1244.1
CF66	**Asn** ^{P8}	*Ac*-**N**ARRRKKRT-NH_2	12.967	1226.7	1227.2
CF67	**Pro** ^{P8}	*Ac*-**P**ARRRKKRT-NH_2	14.330	1209.8	1210.4
CF68	**Gln** ^{P8}	*Ac*-**Q**ARRRKKRT-NH_2	12.817	1240.8	1241.1
CF69	**Ser** ^{P8}	*Ac*-**S**ARRRKKRT-NH_2	13.165	1199.8	1200.1
CF70	**Thr** ^{P8}	*Ac*-**T**ARRRKKRT-NH_2	13.713	1213.8	1214.0
CF71	**Val** ^{P8}	*Ac*-**V**ARRRKKRT-NH_2	16.582	1211.8	1212.0
CF72	**Trp** ^{P8}	*Ac*-**W**ARRRKKRT-NH_2	20.443	1298.8	1298.8
CF73	**Tyr** ^{P8}	*Ac*-**Y**ARRRKKRT-NH_2	16.239	1275.8	1275.8

b) Analogs from the P8-P5 series.

Code	**Modified positions**	**Peptide sequence**	**Retention time[a] t_R [min]**	**Molecular mass**	
				Calculated	**Found[b]**
CF74	**Gly^{P8}Ile^{P5}**	*Ac*-**G**AR**I**RKKRT-NH_2	14.870	1126.7	1126.7
CF75	**Pro^{P8}Ile^{P5}**	*Ac*-**P**AR**I**RKKRT-NH_2	18.244	1166.7	1166.7
CF76	**Gln^{P8}Ile^{P5}**	*Ac*-**Q**AR**I**RKKRT-NH_2	15.723	1197.7	1197.6
CF77	**Ser^{P8}Ile^{P5}**	*Ac*-**S**AR**I**RKKRT-NH_2	15.887	1156.7	1157.0
CF78	**Thr^{P8}Ile^{P5}**	*Ac*-**T**AR**I**RKKRT-NH_2	16.737	1170.7	1170.6
CF79	**Val^{P8}Ile^{P5}**	*Ac*-**V**AR**I**RKKRT-NH_2	19.694	1168.8	1168.7
CF80	**Glu^{P8}Ile^{P5}**	*Ac*-**E**AR**I**RKKRT-NH_2	16.727	1198.7	1199.2

c) Analogs from the P5” series

Code	Modified positions	Peptide sequence	retention time[a] t_R [min]	Molecular mass	
				Calculated	Found[b]
CF81	**Abu^{P5}**	*Ac*-RAR(**Abu**)RKKRT-*NH_2*	12.732	1197.4	1197.2
CF82	**Aib^{P5}**	*Ac*-RAR(**Aib**)RKKRT-*NH_2*	14.368	1197.4	1197.5
CF83	**cLeu^{P5}**	*Ac*-RAR(**cLeu**)RKKRT-*NH_2*	14.999	1223.4	1223.6
CF84	**Nle^{P5}**	*Ac*-RAR(**Nle**)RKKRT-*NH_2*	15.764	1225.5	1225.6
CF85	**Tle^{P5}**	*Ac*-RAR(**Tle**)RKKRT-*NH_2*	15.728	1225.5	1225.6

d) Analogs from P8”-P5” series.

Code	Modified positions	Peptide sequence	Retention time[a] t_R [min]	Molecular mass	
				Calculated	Found[b]
CF86	***D*-Ser^{P8}Abu^{P5}**	*Ac*-**s**AR(**Abu**)RKKRT-*NH_2*	13.999	1128.3	1128.8
CF87	**Ser^{P8}Abu^{P5}**	*Ac*-**S**AR(**Abu**)RKKRT-*NH_2*	15.187	1128.3	1128.7
CF88	**Gly^{P8}Abu^{P5}**	*Ac*-**G**AR(**Abu**)RKKRT-*NH_2*	14.949	1098.3	1098.5
CF89	***D*-Pro^{P8}Abu^{P5}**	*Ac*-**p**AR(**Abu**)RKKRT-*NH_2*	15.115	1138.3	1138.8
CF90	**Pro^{P8}Abu^{P5}**	*Ac*-**P**AR(**Abu**)RKKRT-*NH_2*	15.653	1138.3	1138.5
CF91	***D*-Pro^{P8}**	*Ac*-**p**ARRRKKRT-*NH_2*	14.686	1209.8	1209.6
CF92	***D*-Ser^{P8}**	*Ac*-**s**ARRRKKRT-*NH_2*	11.902	1199.7	1199.7
CF93	***D*-Ile^{P5}**	*Ac*-RAR**i**RKKRT-*NH_2*	15.861	1225.8	1225.9
CF94	**Abu^{P8}Ile^{P5}**	*Ac*-(**Abu**)AR**I**RKKRT-*NH_2*	18.096	1154.4	1154.8
CF95	**Aib^{P8}Ile^{P5}**	*Ac*-(**Aib**)AR**I**RKKRT-*NH_2*	18.934	1154.4	1154.8

e) Cyclic analog.

Code	Modified positions	Peptide sequence	Retention time[a] t_R [min]	Molecular mass	
				Calculated	Found[b]
CF96	**(&)Lys^{P5}Thr$^{P1'}$(&)**	*Ac*-RAR(&)KRKKRT(&)	15.394	1223.8	1223.5

f) azaβ^3-Arg^{P1}Phe$^{P1'}$ analog

Code	Modified positions	peptide sequence	molecular mass	
			calculated	Found[b]
CF97	**(azaβ^3R)^{P1}F$^{P1'}$**	*Ac*-RARRRKK(azaβ^3R)F-*NH_2*	1330.5	1330.0

All analytical HPLC were performed using Shimadzu LC-10A system (column: Jupiter C_{18}, 5 µm, 250×4.6 mm, 300 Å, Phenomenex) with a linear gradient of ACN in H_2O containing 0.1% TFA, 1-30% ACN in 30 min or 2-45% ACN in 25 min, at a flow rate of 1mL/min.

Table S2. Inhibitory activity (K_i) of the selected compounds from the P5 series towards the selected PCs.

Code	Residue at the P5 position	K_i (nM) ± SD			
		furin	PACE4	PC5/6	PC7
CF1	Arg	15 ± 3	13 ± 1	8 ± 3	580 ± 170
CF5	Phe	21 ± 4	34 ± 7	24 ± 3	1330 ± 10
CF8	Ile	6 ± 1	10 ± 5	4.4 ± 1.1	415 ± 40
CF12	Asn	18 ± 5	24 ± 7	17.5 ± 0.1	1600 ± 360

The data in the table are the means ± SD of at least two independent experiments.

Table S3. Enzyme and substrate concentrations used in the present study.

Enzyme	E_0[a] [nM]	Substrate [μM]	K_m[b] [μM]
hFurin	0.55	100	5.04
hPACE4	17.8	100	7.90
hPC5/6	13.7	100	7.16
hPC7	20.7	100	13.45

[a] The used enzyme concentrations in the assay correspond to 2 units for each enzyme at the indicated substrate concentration; the enzyme concentrations in the assay (E_0) were obtained from active-site titration using the inhibitor Dec-Arg-Val-Lys-Arg-CMK. [b] The K_m values for each enzyme were determined in an independent experiment with various substrate concentrations.

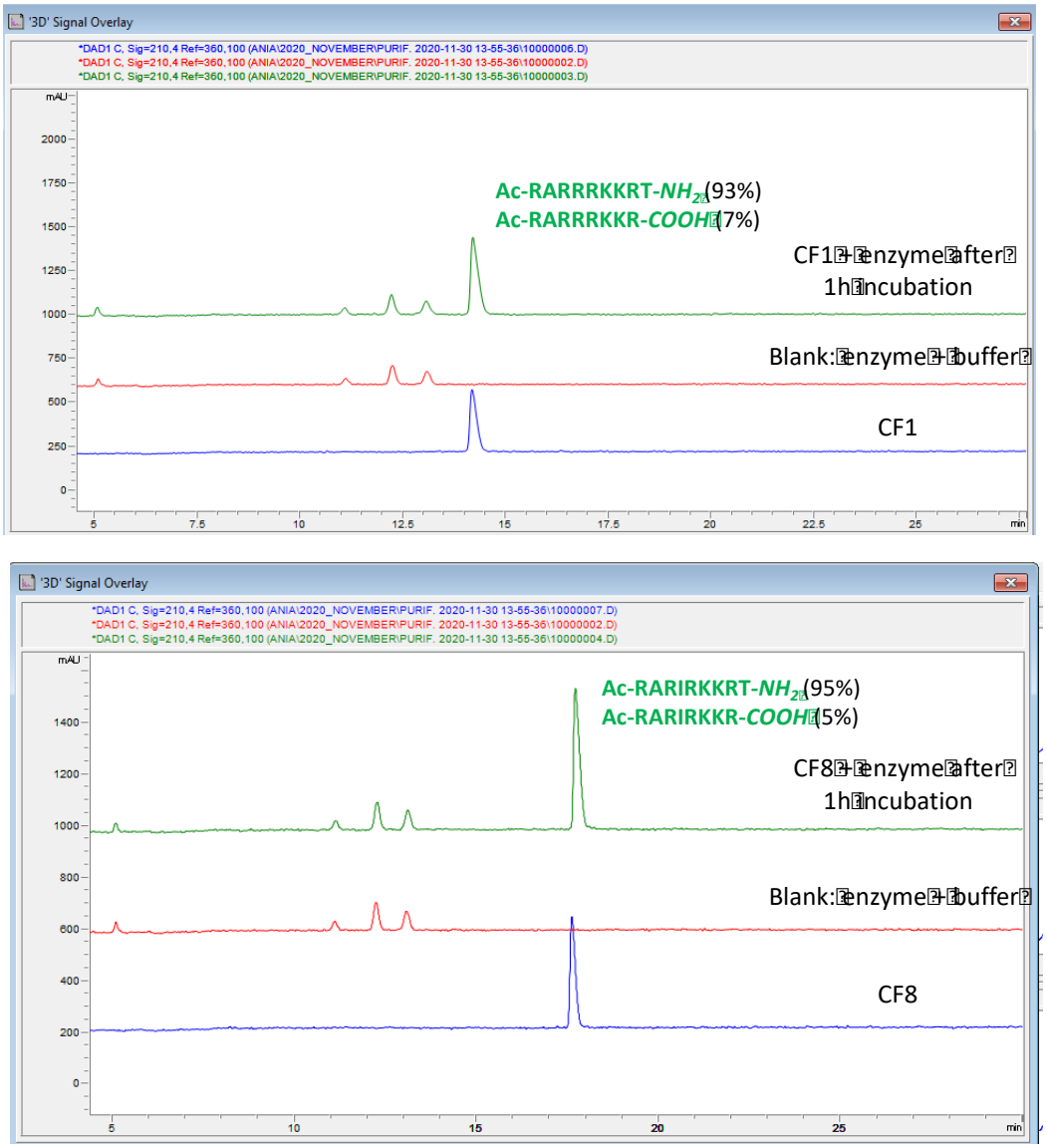

Figure S1. HPLC-HRMS based cleavage studies of CF1 and CF8 inhibitors upon incubation with high amounts of furin.

Despite the presence of the potential furin cleavage sites (e.g., after the P5 and P1 positions) in the structure of the developed inhibitors, only less than < 5 -7% of the found signals can be assigned to the products generated after P1-P1' cleavage. Both compounds (the intact peptide and its cleaved product) elute under the same peak (the elution gradient was 2 - 25% B (0.10% [v/v] TFA in ACN) in A (0.10% [v/v] TFA in H_2O) for 30 min). These results indicate that CF1 and its analogs act as inhibitors not as weak substrates in the kinetic assay. Experimental details: Cleavage reactions were carried out in HEPS buffer (pH 7.5) in a final volume of 100µl, containing 16U of recombinant *h*furin (0.4µl), selected inhibitors (10 µl of 1mM stock solution) and 30µl of BSA solution (6mg of BSA in 1 mL of buffer). The reactions were incubated at 37°C for 1h and stopped by the addition of 30µl of 2% formic acid in H_2O. Samples were cleaned up using centrifuge tube filters and analyzed by HPLC. Peaks collected from the HPLC analysis were lyophilized and analyzed by HRMS.

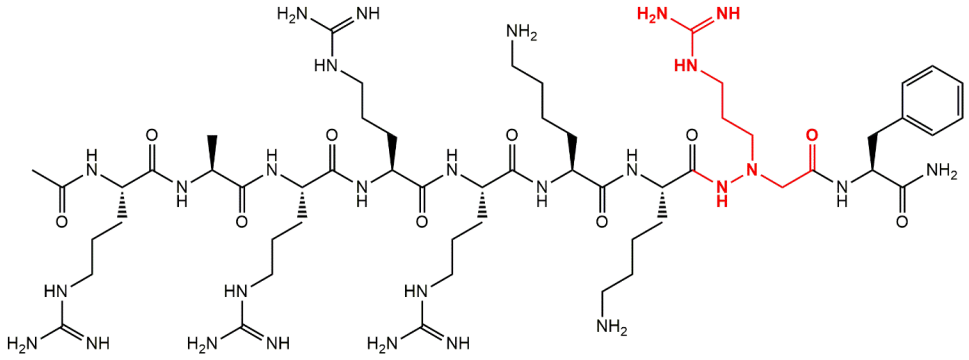

Figure S2. Structure of compound CF97. The azaβ3-Arg is indicated in red.

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