Supplementary Information

The influence of molecular structure on the properties of out-of-equilibrium oscillating enzymatic reaction networks

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S1 General procedures

S1.1 Materials

All chemicals and reagents were used as received from commercial suppliers (*e.g.* Acros, Sigma Aldrich, Ellsworth, Life Technologies) without any further treatment unless stated otherwise. Trypsinogen and trypsin (from bovine pancreas) were purchased from Sigma Aldrich, and aminopeptidase N (EC: 3.4.11.2) was purchased from Novabiochem.

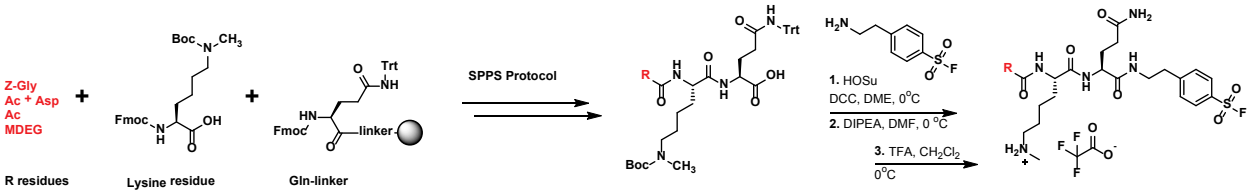
Solvents dimethylformamide (DMF), dimethoxyethane (DME) and dichloromethane (DCM) for synthesis were distilled prior to use by using molecular sieves (4 Å), metallic sodium and calcium hydride (CaH_2), respectively.

S1.2 Instrumentation

Nuclear Magnetic Resonance (NMR) spectra were measured on a *Varian INOVA A-400* spectrometer at 400 MHz for ^{1}H or on a *Bruker-AVANCE III 500* spectrometer at 500 MHz for ^{1}H, 125.8 MHz for $^{13}C(^{1}H)$ and 470.4 MHz for ^{19}F. The chemical shifts for ^{1}H and ^{13}C are given in parts per million (ppm) relative to tetramethylsilane (TMS) and calibrated using a residual peak of the solvent; δ: 3.31 for CD_3OD and δ: 4.79 for D_2O in ^{1}H NMR and δ: 46.7 for CD_3OD in ^{13}C NMR. Multiplets are reported as s (singlet), d (doublet), dd (double doublet), t (triplet), q (quartet) and m (multiplet). Coupling constants are reported as *J* as value in Hertz (Hz). The number of protons (n) for a given resonance is indicated as nH and is based on the spectral integration values. Fourier transform infrared spectroscopy (FT-IR) spectra were recorded on a *Bruker TENSOR 27* spectrometer fitted with an attenuated total reflectance (ATR) cell. Mass spectra were obtained from *Thermo scientific advantage LCQ, JEOL Accurate Time of Flight (ToF)* and *Thermo Finnigan LCQ Fleet instruments*, linear ion trap electrospray ionization (ESI). The masses-to-charge ratio is given in Daltons (Da). *Cetoni® neMESYS, 14.5 gear* high-precision pumps were used for flow experiments. Kinetic measurements with fluorogenic substrates were performed on a *Perkin Elmer LS55* fluorescence spectrometer.

S2 Synthesis

Our approach to synthesize the pro-inhibitor species proceeds by a standard peptide coupling as depicted in the next scheme:



Scheme 2.1 *The synthetic route towards pro-inhibitors* ***1-4****.*

Di-peptides were synthesized using a standard Fmoc solid-phase peptide synthesis (SPPS) protocol on a trityl resin, and subsequently commercially available 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF, **active inhibitor**) was coupled to the carboxylic side. Details of synthesis and characterization of the peptide cores are provided in S2.1, followed by the coupling of inhibitor in S2.2, including full characterization of all individual compounds.

S2.1 Preparation of R functionalized peptide core

5 g Barlos resin (loading 1.6-1.8 mmol/g) was functionalized with α-Fmoc-ε-Trt-L-Glutamine (0.8 mmol/g). *Fmoc-Gln(Trt)-OH* (9 g, 3 eq.) was dissolved in DCM (5 mL) coupled to the resin in presence of *N*,*N*-diisopropylethylamine (DIPEA, 5 mL). After shaking for 4 hours residual linkers on the resin were capped with methanol (MeOH) (1ml for 20 min). The solution was removed after shaking it for 4 hours, and the residue was washed with DCM (3 x 50 mL), DMF (2 x 50 ml) and MeOH (2 x 50 ml) and dried under reduced pressure overnight. Yield: 9.7 g resin (loading of 0.8 mmol/g).

Barlos resin functionalized with α-Fmoc-ε-Trt-L-Glutamine (1 eq.) was swollen in DMF for 20 minutes prior to use. In each of the following steps, Fmoc protecting groups were removed by washing the resin for 20 minutes with 10 mL/g resin 20% piperidine in DMF, and peptide couplings were monitored using standard Kaiser tests until completion was reached.
Lysine residues (1.5 eq.) were dissolved in DMF, and coupled to Gln(Trt)-functionalized resin in presence of diisopropylcarbodiimide (DIPCDI, 3.3 eq.) and *N*-hydroxybenzotriazole (HOBt, 3.6 eq.) for 16 hours. Coupling of *R residues* (MEG, Z-Gly, Ac-Asp, and Ac) differ slightly with respect to each other.

For R=MEG, and ZGly, 3.0 eq. of each were coupled to the peptide block on the resin with diisopropylcarbodiimide (DIPCDI, 3.3 eq.) and *N*-hydroxybenzotriazole (HOBt, 3.6 eq.). The reaction was shaken for 45 minutes.

For R=Ac-Asp, Fmoc-Asp(tBu)-OH (3.0 eq.) was coupled to the peptide block on the resin with diisopropylcarbodiimide (DIPCDI, 3.3 eq.) and *N*-hydroxybenzotriazole (HOBt, 3.6 eq.). The reaction mixture was shaken for 45 minutes. Subsequently, an excess of acetic anhydride and pyridine (>10 eq., 1:1 v/v) was added to the resin.

For R=Ac, the resin was treated with an excess of acetic anhydride and pyridine (>10 eq., 1:1 v/v) directly after the coupling of R_2 modified residues.

After the final coupling, the resin was washed with DMF (3x20 mL), DCM (3x20 mL), methanol (3x20 mL), and again DCM (3x20 mL) before the peptide was cleaved from the resin by applying a mixture of trifluoroethanol (TFE)/acetic acid/DCM (15:15:70, 10 mL/g resin) for 30 minutes. After the addition of hexane (5 x mL), the peptide was concentrated on the rotary evaporator. Lyophilization of the crude product from dioxane yielded the **R-functionalized peptide cores** as white solids, which were used without further purification. Average yield: 85%

S2.2 Pro-inhibitor synthesis

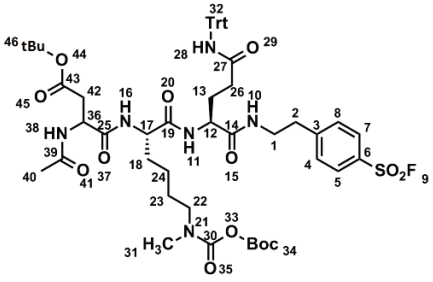
S2.2.1 Side chain protected pro-inhibitor

N,*N*'-dicyclohexylcarbodiimide (DCC) (1 mmol) was added to a stirred solution of **R-functionalized peptide core** (see S2.1) (1 mmol) and *N*-hydroxysuccinimide (HOSu, 1.1 mmol) dissolved in 3 mL distilled dimethoxyethane (DME)/dichloromethane (DCM) solution (1:1) at 0 °C under an argon atmosphere. After allowing the reaction to proceed for one hour at 0 °C, the mixture was stored overnight at 4 °C without stirring. Precipitated dicyclohexyl urea (DCU) was removed by filtration, and the filtrate was dried *in vacuo*.

Subsequently, **Succinimide-activated R-functionalized peptide core** (0.5 mmol, 1 eq.) was dissolved in dry dimethylformamide (DMF, 1 mL) and added dropwise to a stirred solution of 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) (0.55 mmol, 1.1 eq.) and *N*,*N*-diisopropylethylamine (DIPEA, 0.6 mmol, 1.1 eq.) in dry DMF (1 mL) which was cooled to 0 °C. The reaction was carried out under an argon atmosphere and proceeded for 2 hours while the mixture was allowed to slowly reach room temperature. DMF was removed *in vacuo* and the residue was then dissolved in 2 mL potassium bisulfate ($KHSO_4$, 0.1M) and extracted with ethyl acetate (2 x 2 mL). Hereafter, the combined organic phases were washed with water (2 x 2 mL), dried over Na_2SO_4 and concentrated on the rotary evaporator. The residue was purified by column chromatography (SiO_2, CH_2Cl_2/CH_3OH) of which details of eluent composition and yield are reported for the specific pro-inhibitor in the following paragraphs on characterization.

Ac-Asp(tBu)-Lys(Me,Boc)-Gln(Trt)-AEBSF

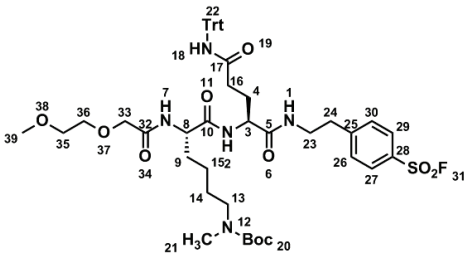
From Ac-Asp(tBu)-Lys(Me,Boc)-Gln(Trt)-OH (63.0 mg, 0.075 mmol), the product could be obtained as a white solid after purification by column chromatography (SiO_2, CH_2Cl_2/CH_3OH 20:1, $R_f \sim 0.4$).
Yield: 36 % (27.6 mg, 0.027 mmol).



^{1}H NMR (400 MHz, CD3OD) δ 7.97 (d, $^3J_{H\text{-}H}$ = 8.4 Hz, 2H, Ar C*H*-5,7), 7.59 (d, $^3J_{H\text{-}H}$ = 8.2 Hz, 2H, Ar C*H*-4,8), 7.31-7.21 (m, 15H, Trt C*H*-32), 4.65 (t, $^3J_{H\text{-}H}$ = 6.8 Hz, 2H, CH_2-36), 4.24 (m, 2H, C*H*-12,17), 3.46 (m, 2H, CH_2-2), 3.22 (m, 2H, CH_2-22), 2.96 (t, $^3J_{H\text{-}H}$ = 6.8 Hz, 2H, CH_2-1), 2.85 (s, 3H, CH_3-31), 2.76 (m, 1H, CH_2-42), 2.60 (m, 1H, CH_2-42), 2.50-2.33 (m, 2H, CH_2-26), 2.11-1.99 (m, 1H, CH_2-13), 1.95 (s, 3H, CH_3-40), 1.90-1.81(m, 2H, CH_2-13,18), 1.77-1.69 (m, 1H, CH_2-18), 1.59-1.50 (m, 2H, CH_2-23), 1.46 (s, 9H, Boc CH_3-34) , 1.44 (s, 9H, ^{t}Bu CH_3-46), 1.41-1.30 (m, 2H, CH_2-24).

MEG-Lys(Me,Boc)-Gln(Trt)-AEBSF

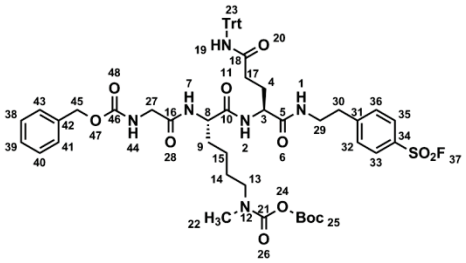
From MEG-Lys(Me,Boc)-Gln(Trt)-OH (151.7 mg, 0.20 mmol), the product could be obtained as a white solid after purification by column chromatography (SiO_2, CH_2Cl_2/CH_3OH 25:1, $R_f \sim 0.4$).
Yield: 55 % (102.6 mg, 0.11 mmol).



^{1}H NMR (400 MHz, CD3OD) δ 7.95 (d, $^3J_{H\text{-}H}$ = 8.5 Hz, Ar C*H*-29,27), 7.56 (d, $^3J_{H\text{-}H}$ = 8.3 Hz, C*H*-26,30), 7.30-7.18 (m, 15H, Trt C*H*-22), 4.27 (m, 1H, C*H*-8), 4.23-4.20 (m, 1H, C*H*-3), 3.91-3.70 (m,2H, CH_2-33), 3.61 (m, 2H, CH_2-35), 3.54 (m, 2H, CH_2-36), 3.45 (t, $^3J_{H\text{-}H}$ = 5.6 Hz, 2H, CH_2-24), 3.38 (s, 3H, CH_3-39), 3.22 (m, 2H, CH_2-13), 2.93 (t, $^3J_{H\text{-}H}$ = 5.2 Hz, 2H, CH_2-23), 2.82 (s, 3H, CH_3-21), 2.49-2.31 (m, 2H, CH_2-16), 2.00-1.89 (m, 1H, CH_2-4), 1.89-1.69 (m, 3H, CH_2-4,9), 1.49-1.62 (m, 2H, CH_2-14), 1.44 (s, 9H, Boc CH_3-20), 1.39-1.28 (m, 2H, CH_2-15).

Z-Gly Lys(Me,Boc)-Gln(Trt)-AEBSF

From Z-Gly-Lys(Me,Boc)-Gln(Trt)-OH (116.0 mg, 0.14 mmol), the product could be obtained as a white solid after purification by column chromatography (SiO_2, CH_2Cl_2/CH_3OH 20:1, $R_f \sim 0.4$).
Yield: 34 % (48.6 mg, 0.048 mmol).



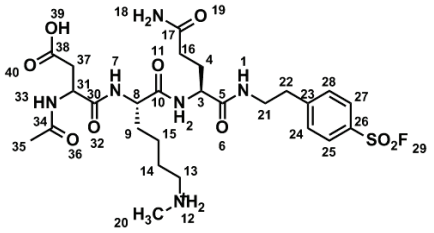
^{1}H NMR (400 MHz, CD3OD) δ 7.93 (d, $^3J_{H\text{-}H}$ = 8.3 Hz, 2H, Ar C*H*-33,35), 7.55 (d, $^3J_{H\text{-}H}$ = 8.1 Hz, 2H, Ar C*H*-32,36), 7.23-7.30 (m, 20H, Trt C*H*-23, Z C*H*-38-43), 5.09 (s, 2H, CH_2-45), 4.31-4.19 (m, 2H, CH_2-3,8), 3.91-3.69 (m, 2H,CH_2-27), 3.48-3.41 (m, 2H, CH_2-30), 3.25-3.05 (m, 2H, CH_2-13), 2.96-2.91(m, 2H, CH_2-30), 2.82 (s, 3H, CH_3-22), 2.42-2.34 (m, 2H, CH_2-17), 2.09-1.98 (m, 1H, CH_2-4) 1.91-1.65 (m, 3H, CH_2-4,9), 1.78-1.59 (m, 3H, CH_2-9,14), 1.44 (s, 9H, Boc CH_3-25), 1.38-1.30 (m, 2H, CH_2-15).

S2.2.1 Pro-inhibitor

The compound was dissolved in a mixture of distilled dichloromethane (DCM) and trifluoroacetic acid (TFA) (1:1, v/v, 2 mL). The solution was stirred at 0 °C for 15 min and for 3 hours at room temperature. Solvents were removed *in vacuo* and the residue was dissolved in 2-propanol (~0.2 mL). The product was precipitated using diethyl ether (Et_2O, 2 mL). After removal of Et_2O, CH_2Cl_2 (1 mL) was added to the precipitate, and the suspension was stirred for 30 minutes to wash the product. Removal of CH_2Cl_2 yields a glassy product, which could be precipitated using Et_2O (2 mL). Removal of Et_2O yields the TFA salt of pro-inhibitor as a white solid.

2: *Ac-Asp-Lys(Me)-Gln-AEBSF*

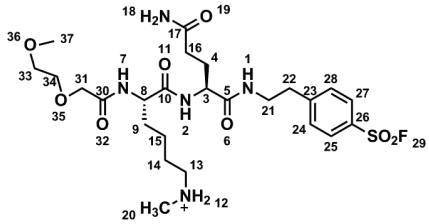
Yield: 89% (15.7 mg, 0.021 mmol).



FT-IR (cm^{-1}): 3296 (ν_{NH}), 1661 (ν_{CO}), 1404 (ν_{SO}), 1215 (ν_{SO}); ^{1}H NMR (400 MHz, CD3OD) δ 7.96 (d, $^3J_{H\text{-}H}$ = 8.4 Hz, 2H, Ar C*H*-25,27), 7.61-7.56 (d, $^3J_{H\text{-}H}$ = 8.0 Hz, 2H, Ar C*H*-24,28), 4.65 (t, $^3J_{H\text{-}H}$ = 6.8 Hz, 1H, *CH*-31), 4.24-4.17 (m, 2H, CH_2-3,8), 3.55-3.41 (m, 2H, CH_2-22), 3.09-2.95 (m, 4H, C*H*-21,13), 2.94-2.85 (m, 1H, CH_2-37), 2.78 (m, 1H, CH_2-37), 2.71 (s, 3H, CH_3-20), 2.26 (t, $^3J_{H\text{-}H}$ = 7.6 Hz, 2H, CH_2-16), 2.10-2.01 (m, 1H, CH_2-4), 1.99 (s, 3H, CH_3-35), 1.98-1.84 (m, 1H, CH_2-4), 1.79-1.60 (m, 3H, CH_2-9,14), 1.59-1.41 (m, 2H, CH_2-15); LCQMS-ESI (Da): *m/z* observed 631.4 for $C_{18}H_{27}FN_3O_6S^+$ $[M]^+$; *m/z* calculated for $[M]^+$: 631.25.

3: *MEG-Lys(Me)-Gln-AEBSF*

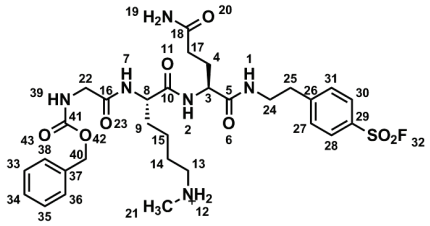
Yield: 75% (64.3 mg, 0.091 mmol).



FT-IR (cm^{-1}): 3293 (ν_{NH}), 1658 (ν_{CO}), 1413 (ν_{SO}), 1214 (ν_{SO}); ^{1}H NMR (400 MHz, CD3OD) δ 7.98 (d, $^3J_{H\text{-}H}$ = 8.4 Hz, Ar C*H*-25,27), 7.60 (d, $^3J_{H\text{-}H}$ = 8.3 Hz, Ar C*H*-24,28), 4.37-4.33 (m, 1H, C*H*-8), 4.28-4.21 (m, 1H, C*H*-3), 4.02 (s, 2H, CH_2-31), 3.76-3.66 (m, 2H, CH_2-33), 3.59 (t, $^3J_{H\text{-}H}$ = 3.6 Hz, 2H, CH_2-34), 3.51(t, $^3J_{H\text{-}H}$ = 5.2 Hz, 2H, CH_2-22), 3.40 (s, 3H, CH_3-37), 3.05-2.92 (m, 4H, CH_2-13,21), 2.99 2.69 (s, 3H, CH_3-20), 2.24 (t, $^3J_{H\text{-}H}$ = 5.6 Hz, 2H, CH_2-16), 2.05-1.94 (m, 1H, CH_2-4), 1.91-1.82 (m, 2H, CH_2-9), 1.78-1.66 (m, 3H, CH_2-4,14), 1.51-1.41 (m, 2H, CH_2-15); LCQMS-ESI (Da): *m/z* observed 590.4 for $C_{26}H_{40}FN_6O_9S^+$ $[M]^+$; *m/z* calculated for $[M]^+$: 590.27.

4: *Z-Gly-Lys(Me)-Gln-AEBSF*

Yield: 75% (21.0 mg, 0.027 mmol).



FT-IR (cm^{-1}): 3293 (ν_{NH}), 1668 (ν_{CO}), 1414 (ν_{SO}), 1215 (ν_{SO}); ^{1}H NMR (400 MHz, CD3OD) δ 7.97 (d, $^3J_{H\text{-}H}$ = 8.4 Hz, 2H, Ar C*H*-28,30), 7.57(d, $^3J_{H\text{-}H}$ = 8.3 Hz, 2H, Ar C*H*-27,31), 7.37-7.26 (m, 4H, Z C*H*-33-38), 5.08 (s, 2H, CH_2-40), 4.27-4.19 (m, 2H, C*H*-3,8), 3.86-3.71 (m, 2H, CH_2-22), 3.52-3.40 (m, 2H, CH_2-25), 3.01-2.89 (m, 4H, CH_2-13,24), 2.67 (s, 3H, CH_3-21), 2.19-2.22 (m, 2H, CH_2-17), 2.07-1.97 (m, 1H, CH_2-4), 1.92-1.81 (m, 2H, CH_2-9), 1.72-1.63 (m, 3H, CH_2-4,14), 1.52-1.38 (m, 2H, CH_2-15); LCQMS-ESI (Da): *m/z* observed 667.3 for $C_{18}H_{26}FN_3O_6S^+$ $[M+H]^+$; *m/z* calculated for $[M+H]^+$: 666.3.

S3 Kinetic studies

Kinetic studies were performed to determine the rate constants of pro-inhibitor cleavage by trypsin, degradation, and background inhibition of pro-inhibitor in isolated reactions. All rate constants for pro-inhibitor **1** and for all reactions not involving pro-inhibitor have been reported before (see ref. 17 of main text). A summary of the kinetic studies is presented at the end of this paragraph and contains all determined rate constants.

S3.1 Pro-inhibitor cleavage by trypsin

The activation step (cleavage of pro-inhibitors by *Tr*) was tested by mixing varying concentrations of pro-inhibitor with *Tr* and measuring the conversion by ^{1}H-NMR. Typically, $[Tr]_0$ was tuned to obtain major conversion levels within the first 20-30 minutes of measurement, in order to minimize the influence of unavoidable background reactions that are insignificant at this timescale (more details follow in S3.2). Enzymatic reactions were carried out in 100 mM Tris-HCl, pD 7.7, 20 mM $CaCl_2$ in D_2O at 23 °C. The initial speed of the reaction was typically measured between four and nine minutes after starting the experiment. Finally, the inverse of the initial speed of the reaction is plotted against the inverse of the pro-inhibitor concentration in a Lineweaver-Burk plot. A linear fit of this plot yields the slope (K_M/V_{max}) and the y-axis intercept ($1/V_{max}$) from which K_M and k_{cat} ($V_{max} = [Tr]_0 \times k_{cat}$) are calculated. Results from the different experiments are shown below in Figure S3.1.

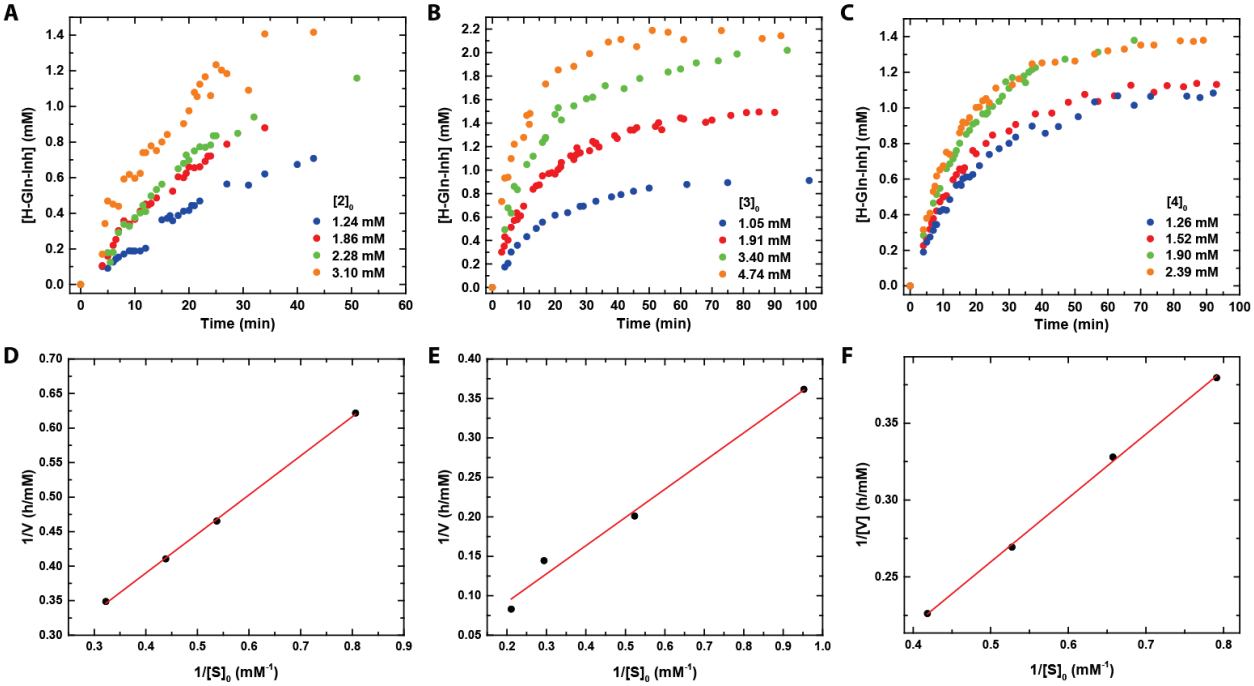


Figure S3.1. *Determination of pro-inhibitor activation rate. (a) Pro-inhibitor **2**, $[Tr]_0$ = 86 µM, (b) Pro-inhibitor **3**, $[Tr]_0$ = 100 µM, (c) Pro-inhibitor **4**, $[Tr]_0$ = 5 µM. (D-F) Lineweaver-Burk plots for pro-inhibitors **2**-**4**, respectively. Solid lines denote linear fits from which the kinetic constants are derived.*

S3.2 Background reactions

S3.2.1 Background inhibition of trypsin

Even though the amine of 4-(2-aminoethyl)benzenesulfonyl fluoride, that ensures recognition for *Tr*, is masked in the pro-inhibitor, background inhibiton of *Tr* can still occur through non-specific interactions. To measure this background inhibition, *Tr* (43 µM) is mixed with pro-inhibitor (258 µM) in 100 mM Tris-HCl, pH 7.7, 20 mM $CaCl_2$ at room temperature. Aliquots of the reaction mixture are quenched in 0.1 M $KHSO_4$ and analyzed by the standard fluorogenic assay (See experimental section of main text). The reaction is treated as a second-order bimolecular process, in which other reactions (pro-inhibitor degradation and cleavage) are ignored. This process follows the equation:

$$\ln\frac{[Tr]\times[Pro]_0}{[Tr]_0\times[Pro]} = -([Pro]_0 - [Tr]_0)\times k_{inh}\times t \qquad \textit{(Eq. S1)}$$

with $[Tr]_0$ and $[Pro]_0$ the starting concentrations of *Tr* and pro-inhibitor, respectively, and k_{inh} the inhibition rate constant. All variables are known or are measured ($[Tr]$) apart from $[Pro]$. However, this term can be rewritten, since the amount of pro-inhibitor that has reacted at time *t* must equal the amount of inhibited *Tr*, or in mathematical terms:

$$[Pro] = [Pro]_0 - ([Tr]_0 - [Tr]) \qquad \textit{(Eq. S2)}$$

When we insert this into Equation S1, we obtain:

$$\ln\frac{[Tr]\times[Pro]_0}{[Tr]_0\times([Tr]+[Pro]_0-[Tr]_0)} = -([Pro]_0 - [Tr]_0)\times k_{inh}\times t \qquad \textit{(Eq. S3)}$$

Now, we have an equation with only one unknown variable (k_{inh}), and a linear fit of the natural logarithm in Equation S3 versus time has a slope of $-k_{inh}\times([Pro]_0 - [Tr]_0)$ from which the rate constant is calculated. Results from the different experiments are shown below in Figure S3.2.

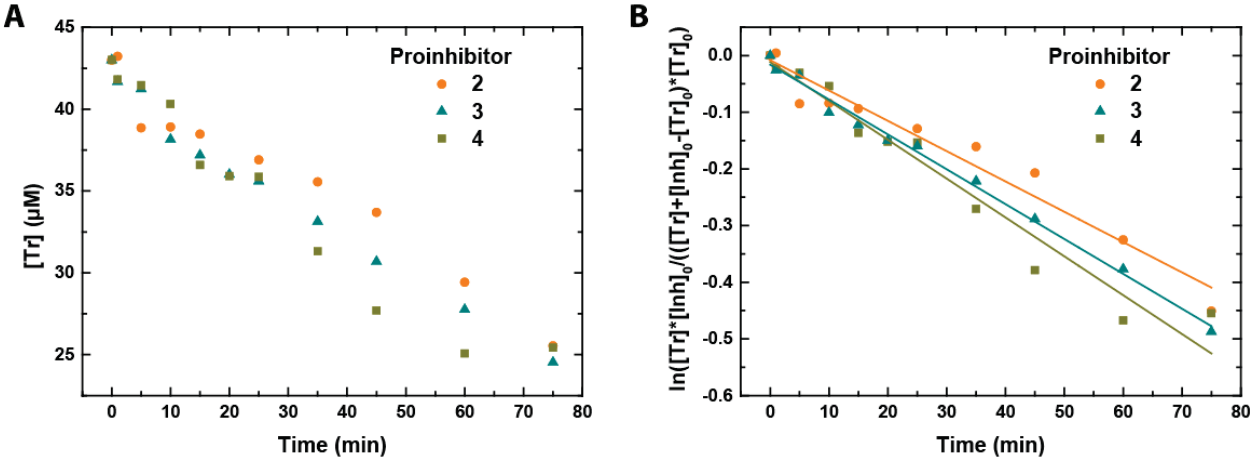


Figure S3.2. *Determination of background inhibiton rate by pro-inhibitors* ***2-4****. A) Decrease in Tr activity over time. B) The transformation that yields the rate constants through linear fits (solid lines).*

S3.2.2 Degradation of pro-inhibitors by sulfonyl fluoride side reactions

In aqueous solutions, the sulfonyl fluoride moiety of the pro-inhibitor is prone to hydrolyze, resulting in a species that cannot inhibit *Tr*. To measure its degradation rate by ^{1}H-NMR, pro-inhibitors (1-1.5 mM)

were dissolved in 100 mM Tris-HCl, pD 7.7, 20 mM $CaCl_2$ in D_2O at 23 °C. Since water is in excess, we treat the reaction as a pseudo-first order process. The natural logarithm of the ratio between the concentration of pro-inhibitor and the starting concentration yields a line with a slope that equals $-k_{degr}$. Results from the different experiments are shown below in Figure S3.3.

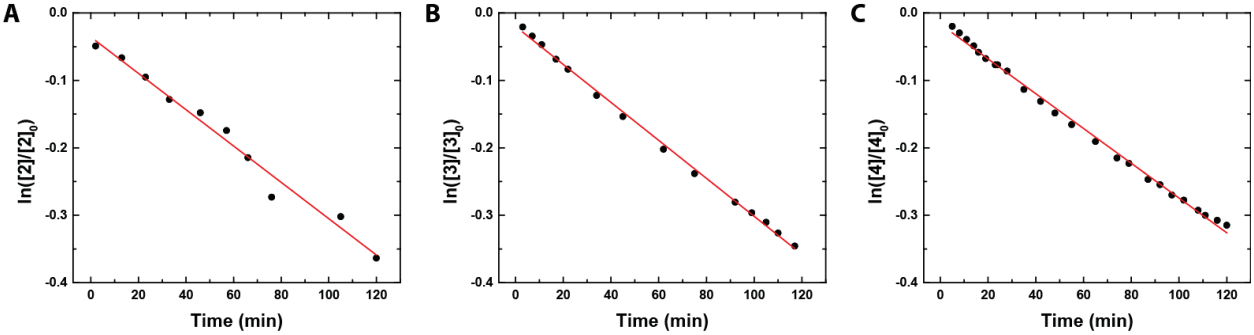


Figure S3.3. *Determination of rate of degradation of pro-inhibitors* ***2*** *(A),* ***3*** *(B), and* ***4*** *(C). The red lines denote linear fits to the experimental data points from which the kinetic constants are derived.*

S3.3 Summary of kinetic studies

Pro-inhibitor	N-terminal R-group	k_{cat} (h^{-1})	K_M (mM)	k_{cat}/K_M ($mM^{-1}\ h^{-1}$)	k_{inh} ($mM^{-1}\ h^{-1}$)	k_{deg} (h^{-1})
1[a]	Acetyl	1027	21.9	47	1.76	0.18
2	Ac-Asp	71	3.5	21	1.49	0.16
3	MEG	487	17.4	28	1.72	0.17
4	Z-Gly	3844	8.0	482	2.21	0.16

[a] Rate constants from ref. 17 of main text.

S4 Computational screening analysis

This section describes the bifurcation analysis and the computational screening process for retrieving the initial conditions in our flow experiments. Both methods use the previously reported mathematical model based on mass action kinetics (see reference 17 of the main text), and share general build-up (see S4.1). S4.2 describes in detail how we have used this general setup to perform the bifurcation analysis, followed by details and results of the screening program in S4.3. The scripts of both methods are included in S4.4. The reader is referred to supplementary information in reference 17 of the main text for details on the wave classifier and mass action model assumptions.

S4.1 End-state analysis

The dynamical behavior of the CRN can be characterized by an end-state analysis. In typical screenings for oscillatory regimes, we use this strategy to assess in which regime of the screened parameter [Tr] oscillates. As depicted in Figure S4.1, the mathematical model is used to simulate time series at different conditions.

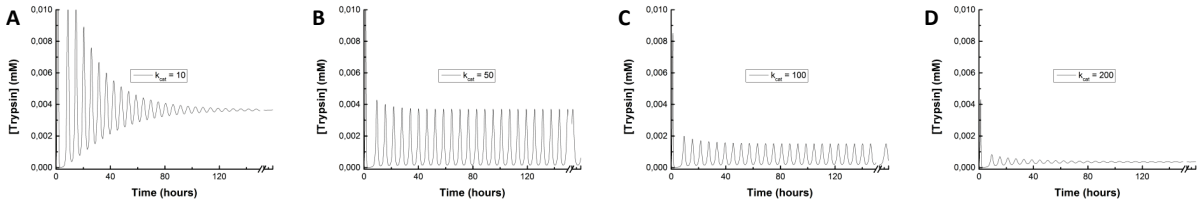


Figure S4.1: *Simulated time series with different identical initial conditions while varying one parameter, in this case activation rate. The end-points in* ***(A-D)*** *are recorded at t=1000 h, and represent the final state of the system.*

The separated figures show how the network responds when changing activation rates, k_{act}. Noteworthy, the behaviors are very different for the different conditions. However, in the end-state analysis we solely pay attention to the concentration changes of trypsin after 1000 hours, representing $[Tr]_{(t=\infty)}$, and classify the end-states as either being; steady state or oscillatory.

General structure of scripts

The scripts used for the analyses in S4.2, and in S4.3 share a common structure; that is, (1) assemble required input data, (2) run core simulations and analysis, and finally produce (3) output of results. Depending on the type of analysis, input data is composed of fixed initial conditions, and rate constants. Non-fixed conditions are varied within the defined borders of the parameter of interest. Core simulations are based on integrations of differential equations describing the dynamics of the enzymatic CRN at each of the varied parameter. Using the previously reported wave classifier, we analyze the end-state of the system.

S4.2 Bifurcation analysis

S4.2.1 Description

The bifurcation analysis was carried out as described in the main text. The phase diagram is constructed from 300 simulated time series in which k_{act} (i.e. k_{cat}/K_M) was varied from 0-500 $mM^{-1}h^{-1}$.

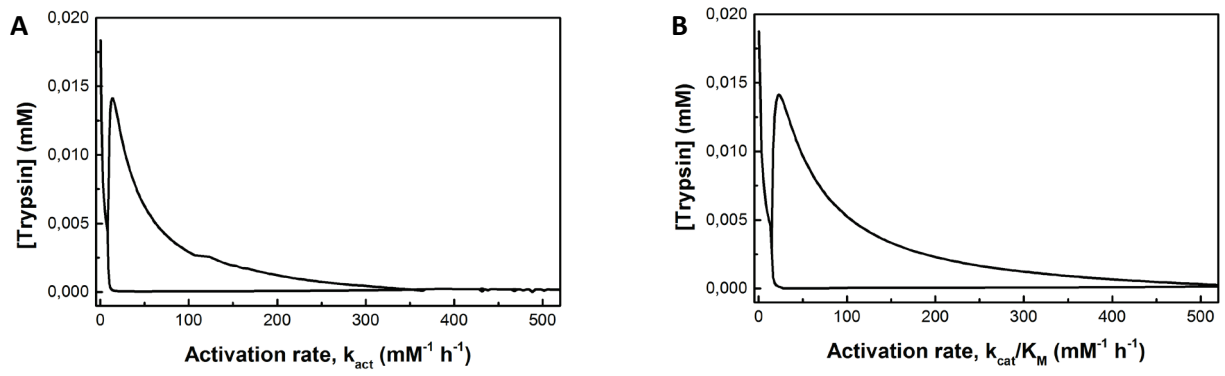


Figure S4.2. *Bifurcation diagrams of activation rate, which could be expressed in either k_{act} or k_{cat}/K_M, in which $K_M = \frac{k_{cat} + k_r}{k_f}$. Under fixed k_{cat} , variations in activation rate are achieved by either changing k_f* ***(A)*** *or k_r* ***(B)****. Fixed initial conditions are: Tg (0.167 mM), Tr (0.00023 mM), Ap (0.11 U mL^{-1}), pro-inhibitor (1.5 mM), flow/Volume (0.2 h^{-1}), optimized rate constants (as reported in ref. 17 of the main text).*

The kinetic studies allow experimental determination of kinetic parameters, k_{cat}, K_M. However, the mass-action based mathematical model, requires to be written in the form of isolated reactions including k_f and k_r, which are experimentally inaccessible. Based on physical-chemical considerations of how the substituents influence the chemical binding and catalytic efficiency of the pro-inhibitor cleavage, we fixed k_{cat} in our bifurcation analysis. This reduces the variable parameters to the two possibilities shown in Figure S4.2, in which k_f and k_r are calculated according the following equations:

$$k_f = k_{act} * \frac{k_r + k_{cat}}{k_f} \qquad \textit{(Eq. S4)}$$

$$k_r = k_{cat} * \left(\frac{k_f}{k_{act}} - 1\right) \qquad \textit{(Eq. S5)}$$

Figure S4.2 shows that the differences in varying either k_f or k_r are insignificant.

S4.2.2 Bifurcation analysis script

```
function [t,x]=Bifurcation_comm_paper_vma(act_min,act_max,stepsize,constanttype,Ap,rate_type)

    % Take data from excel and transform it into 1structure containing all
    % required information to run this script.
    load    input.mat

    % Variable space
        if isempty(act_min)
            act_min=0.1;
        end

        if isempty(act_max)
            act_max=1000;
        end

    % Sampling size
        % if stepsize is 1, then sampling size is determined by ratio max/min.

        if isempty(stepsize)
            stepsize=1;
        end
        act_space = linspace(act_min,act_max,stepsize);
```

```
    % TEMPORARY SAVE SPACE
    l_j= length(act_space(1,:));
            result_Up = zeros(l_j,1);
            result_Low= zeros(l_j,1);

    % MODEL time
        % reaction time can be changed, but keep at least at 1000 for final analysis.
        timespan            = [0:0.1:1000];

    % MODEL rate parameters
        % Constanttype is set of rate constants which are derrived in different
        % ways, but all of them work.
        if  constanttype == 0;
            % 0 Measured rate constants
            % See [Semenov, Wong; nchem 2015] S3.3 Table S1
                kset    = [parscreening{:,4}];
        end

        if  constanttype == 1;
            % 1 for Optimized rate constants by modified genetic algoritmh
            % See [Semenov, Wong; nchem 2015] S4.3.2 Table S3
                kset    = [parscreening{:,6}];
        end


   % Rate constants assembled for model is based on the choice of
    % constanttype.
            k.kf_auto_tr    =   kset(1);
            k.kr_auto_tr    =   kset(2);
            k.kc_auto_tr    =   kset(3);
            k.kf_act        =   kset(4);
            k.kr_act        =   kset(5);
            k.kc_act        =   kset(6);
            k.kc_delay      =   kset(7);
            k.Km_delay      =   kset(8);
            k.kf_inh_i      =   kset(9);
            k.kf_inh_ai     =   kset(10);
            k.kf_inh_aai    =   kset(11);
            k.kf_degr_i     =   kset(12);
            k.kf_degr_ai    =   kset(13);
            k.kf_degr_aai   =   kset(14);
            k.kc_auto_tg    =   kset(15);
                 kflow = 0.20;  % 50uL h-1

%% NUMERICAL INTEGRATION BASED ON IRREVERSIBLE INHIBITION:

        % INITIAL CONCENTRATION of the system
            xo      = [species{1:8,5}];
            if isempty(Ap)
                    xo(4)   = 25;
            else
                    xo(4) = Ap;
            end


%% BIFURCATION ANALYSIS    for act_space=kc*kf/(kr+kc)
tic % Start the clock


            if rate_type == 'r'; disp('kr varied')
                % Change Km based on kr
                    kr_space = ((1./act_space)*k.kf_act-1)*k.kc_act;
                    for j = 1:l_j;
                    disp(j);
                    k.kr_act = kr_space(j);
                        % INTERGRATION
                        OPTIONS             = [];
                        [t,x] = ode23t(@dnf_trypsin_irrev, timespan, xo, [], k, kflow, xo);


                        % CHECKSUS ANALYSIS
                        % detect Tr
                            [~,~,SR] = checksus321(t,x(:,2),0.001);
                            if ~islogical(SR)
                                    if SR{4,3} > 0.0001 % define threshold under which we assume no regime.
                                        result_Up(j) = double(SR{end,3}); % min
```

```
                                result_Low(j) = double(SR{end,4});
                            end
                        else    result_Up(j) = x(end,2); % ss
                                result_Low(j) = x(end,2); % ss
                        end

                    end

                elseif rate_type == 'f'; disp('kf varied')
                % Change Km based on kf
                    kf_space = (act_space*(k.kr_act+k.kc_act)./k.kf_act);

                    for j = 1:l_j;
                    disp(j);
                    k.kf_act = kf_space(j);
                        % INTERGRATION
                        OPTIONS             = [];
                        [t,x] = ode23t(@dnf_trypsin_irrev, timespan, xo, [], k, kflow, xo);


                        % CHECKSUS ANALYSIS
                        % detect Tr
                            [~,~,SR] = checksus321(t,x(:,2),0.001);
                            if ~islogical(SR)
                                    if SR{4,3} > 0.0001 % define threshold under which we assume no regime.
                                        result_Up(j) = double(SR{end,3}); % min
                                        result_Low(j) = double(SR{end,4});
                                    end
                                else    result_Up(j) = x(end,2); % ss
                                        result_Low(j) = x(end,2); % ss
                                end

                    end

            end

%% FINAL SAVES
        % 1=ss if exist, 2=low, 3=high

        save(['result_bifurcation_activation_Ap' num2str(Ap) 'kf.mat'],'act_space','result_Up','result_Low')

toc; timing = toc; Time_run=timing/3600; Model_run=(l_j);
save('Modelling_data.mat','Time_run','Model_run')


%% PLOT
figure
    plot (act_space, result_Low, '-k',...
                                    'LineWidth',2, ...
                                    'LineSmoothing','on',...
                                    'MarkerEdgeColor','g',...
                                    'MarkerFaceColor',[1 1 1],...
                                    'MarkerSize',5);  hold on;
    plot (act_space, result_Up, '-k',...
                                    'LineWidth',2, ...
                                    'LineSmoothing','on',...
                                    'MarkerEdgeColor','g',...
                                    'MarkerFaceColor',[1 1 1],...
                                    'MarkerSize',5); hold on;

end
```

S4.2 3D plots and calculations

S4.3.1 Description

The screening procedure for optimal conditions was described in detail in the main text. Here, we want to add details on the simulations required for the output shown in Figure 3 and 4 of the main text. We used the kinetic constants reported in S3.3 for each pro-inhibitor. In our analysis we assumed that K_M could be

reduced to k_r, under fixed k_f (see script section `%% 1-B`). Then, simulations were performed for each combination of parameters in the varied 3D space. The end-state of each grid is analyzed for a sustained regime at different amplitude thresholds (0.0025, 0.005, 0.0075, and 0.010 mM, see script section `%% 1-C`). Dividing these results over the size of each *(flow,Ap)* plane in the 3D space yields the plots shown in Figure 4b of the main text. The analyzed grids could also be saved as data-cubes, and with the use of the isosurface function in Matlab®, the data at different thresholds is projected in 3D phase plots.

S4.3.2 D plot and optimal condition screening script

```
function [t,x]=Screening_phps_comm_paper_v4(constanttype,proinhibitor,stepsizePI,stepsizeA)

    % Take data from excel and transform it into 1structure containing all
    % required information to run this script.
    load    input.mat

%% 0 MODEL Requirements
    % MODEL reaction time for end-state
        % reaction time can be changed, but keep at least at 1000 for final analysis.
        timespan    = [0:0.1:1000];

    % MODEL Initial concentrations of the system
        xo      = [species{1:8,5}];

    % MODEL rate parameters
        % Constanttype is set of rate constants which are derrived in different
        % ways, but all of them work.

            if  constanttype == 0;
                % 0 Measured rate constants
                % See [Semenov, Wong; nchem 2015] S3.3 Table S1
                    kset    = [parscreening{:,4}];
            end

            if  constanttype == 1;
                % 1 for Optimized rate constants by modified genetic algoritmh
                % See [Semenov, Wong; nchem 2015] S4.3.2 Table S3
                    kset    = [parscreening{:,6}];
            end


        % Rate constants assembled for model is based on the choice of
        % constanttype.

            k.kf_auto_tr    =   kset(1);
            k.kr_auto_tr    =   kset(2);
            k.kc_auto_tr    =   kset(3);
            k.kf_act        =   kset(4);
            k.kr_act        =   kset(5);
            k.kc_act        =   kset(6);
            k.kc_delay      =   kset(7);
            k.Km_delay      =   kset(8);
            k.kf_inh_i      =   kset(9);
            k.kf_inh_ai     =   kset(10);
            k.kf_inh_aai    =   kset(11);
            k.kf_degr_i     =   kset(12);
            k.kf_degr_ai    =   kset(13);
            k.kf_degr_aai   =   kset(14);
            k.kc_auto_tg    =   kset(15);
                if  proinhibitor == 1
                    % Ac
                    k.Km_act        =   21.9;
                    k.kf_act        =   kset(4);
                    k.kc_act        =   1027;
                    k.kr_act        =   k.Km_act*k.kf_act-k.kc_act;
                    disp('Ac')
                elseif proinhibitor == 2
                    %ZGly
                    k.Km_act        =   8.0;
                    k.kf_act        =   kset(4);
                    k.kc_act        =   3844;
                    k.kr_act        =   k.Km_act*k.kf_act-k.kc_act;
                    disp('ZGly')
```

```
            elseif proinhibitor == 3
                %Asp
                k.Km_act        =   3.5;
                k.kf_act        =   kset(4);
                k.kc_act        =   71;
                k.kr_act        =   k.Km_act*k.kf_act-k.kc_act;
                disp('AcAsp')
            elseif proinhibitor == 4
                %DEG
                k.Km_act        =   17.4;
                k.kf_act        =   kset(4);
                k.kc_act        =   487;
                k.kr_act        =   k.Km_act*k.kf_act-k.kc_act;
                disp('MEG')
            end
%%  1-A SCREENING A (1X40)
    % Variable space

    % Spaces for 3d volume are defined:
    Tg_0=0.167; % initial conditions for Tg
            if isempty(stepsizePI)
                stepsizePI=1;
            end
    PI_space=linspace(0,5,stepsizePI);
    Tg_space=Tg_0;


    % Define lengths of spaces:
            l_g= length(Tg_space);
            l_h= length(PI_space);
        tic;
            for g = 1:l_g;
                xog         = xo;

                Tg_spaceg = Tg_space;
                xog(1)      = Tg_spaceg(g);

                for h = 1:l_h;

                    PI_spaceh = PI_space;
                    xog(3)      = PI_spaceh(h);

%% 1-B CORE DIMENSIONS for analysis Phps

                    % Sampling size

                        if isempty(stepsizeA)
                            stepsizeA=1;
                        end

                    % Spaces for 3d volume are defined:
                        flow_space  = linspace(0,150,stepsizeA);
                        Apep_space  = linspace(0,75,stepsizeA);

                        % Create save space
                            result1 = zeros(length(flow_space),length(Apep_space));
                            result2 = zeros(length(flow_space),length(Apep_space));
                            result3 = zeros(length(flow_space),length(Apep_space));
                            result4 = zeros(length(flow_space),length(Apep_space));

                        % Define lengths of spaces:
                            l_i= length(flow_space);
                            l_j= length(Apep_space);


                            % Ground phase (f,T)

                            for i = 1:l_i;  disp([g h i]);


                                flow_spacei = flow_space;

                                kflow       = flow_spacei(i)/250;
                                k.kflow     = kflow;
                                xoi         = xog;
```

```
                    for j = 1:l_j;

                        Apep_spacej = Apep_space;
                        xoi(4)      = Apep_spacej(j);

                    % MODEL run
                        % within each looped m,i,j. (thus every grid)

                        [t,x] = ode23t(@dnf_trypsin_irrev, timespan, xoi, [], k, kflow, xoi);

                    % CHECKSUS ANALYSIS
                        [~,~,SR] = checksus321(t, x(:,2), 0.001);
                        % 0.001 threshold For MM necessary otherwise recognize too many peaks
                            if ~islogical(SR)
                                if SR{4,3} > 0.0025
                                    [amp,per] = checksusphase(t,x(:,2),0.003,1);
                                    result1(i,j) = amp;
                                end
                                if SR{4,3} > 0.005
                                    [amp,per] = checksusphase(t,x(:,2),0.003,1);
                                    result2(i,j) = amp;
                                end
                                if SR{4,3} > 0.0075
                                    [amp,per] = checksusphase(t,x(:,2),0.003,1);
                                    result3(i,j) = amp;
                                end
                                if SR{4,3} > 0.010
                                    [amp,per] = checksusphase(t,x(:,2),0.003,1);
                                    result4(i,j) = amp;
                                end
                            end
                        end
                    end
                    Apep_space=Apep_space*0.010637;
                save(['php' num2str(g) num2str(h) '.mat'], 'result1', 'result2', 'result3',
                'result4', 'flow_space', 'Apep_space')
%% 1-C DETECT OPTIMUM
                P=size(result1); % size of matrix
                W_1=sum(sum(nnz(result1))); % #filled above set threshold
                resultA(g,h)=100*W_1/(P(1)*P(2)); % Percentage of coverage
                Q=size(result2); % size of matrix
                W_2=sum(sum(nnz(result2))); % #filled above set threshold
                resultB(g,h)=100*W_2/(Q(1)*Q(2)); % Percentage of coverage
                R=size(result3); % size of matrix
                W_3=sum(sum(nnz(result3))); % #filled above set threshold
                resultC(g,h)=100*W_3/(R(1)*R(2)); % Percentage of coverage
                S=size(result4); % size of matrix
                W_4=sum(sum(nnz(result4))); % #filled above set threshold
                resultD(g,h)=100*W_4/(S(1)*S(2)); % Percentage of coverage

            end
        end
            plot(PI_space,resultA,'-','Color', [0.9 0.9 0.9], 'LineWidth',2); hold on;
            plot(PI_space,resultB,'-','Color', [0.6 0.6 0.6], 'LineWidth',2);
            plot(PI_space,resultC,'-','Color', [0.3 0.3 0.3], 'LineWidth',2);
            plot(PI_space,resultD,'-','Color', [0.1 0.1 0.1], 'LineWidth',2);
               xlabel(['[Pro inh' num2str(proinhibitor) ']'],'FontSize',10, 'FontName','Calibri');
               ylabel('%','FontSize',10, 'FontName','Calibri');
               title(  'screening',...
                   'FontWeight','bold','FontSize',12,'FontName','Helvetica');

toc; timing = toc; Time_run=timing/3600; Model_run=(l_g*l_h*l_i*l_j);
save(['Screening' num2str(proinhibitor)
'.mat'],'resultA','resultB','resultC','resultD','Tg_space','PI_space','Time_run','Model_run');
```

```
%% 2 3D plot


        if  proinhibitor == 1
            % Ac
            load Screening1.mat;
            disp('Ac')
        elseif proinhibitor == 2
            %ZGly
            load Screening2.mat;
            disp('ZGly')
        elseif proinhibitor == 3
            %Asp
            load Screening3.mat;
            disp('AcAsp')
        elseif proinhibitor == 4
            %DEG
            load Screening4.mat;
            disp('MEG')
        end

% %% Build volume of data 3D
load php11.mat;     threshold_003(:,:,1)=result1';  threshold_005(:,:,1)=result2';
threshold_008(:,:,1)=result3';  threshold_010(:,:,1)=result4';
load php12.mat;     threshold_003(:,:,2)=result1';  threshold_005(:,:,2)=result2';
threshold_008(:,:,2)=result3';  threshold_010(:,:,2)=result4';
load php13.mat;     threshold_003(:,:,3)=result1';  threshold_005(:,:,3)=result2';
threshold_008(:,:,3)=result3';  threshold_010(:,:,3)=result4';
load php14.mat;     threshold_003(:,:,4)=result1';  threshold_005(:,:,4)=result2';
threshold_008(:,:,4)=result3';  threshold_010(:,:,4)=result4';
load php15.mat;     threshold_003(:,:,5)=result1';  threshold_005(:,:,5)=result2';
threshold_008(:,:,5)=result3';  threshold_010(:,:,5)=result4';
load php16.mat;     threshold_003(:,:,6)=result1';  threshold_005(:,:,6)=result2';
threshold_008(:,:,6)=result3';  threshold_010(:,:,6)=result4';
load php17.mat;     threshold_003(:,:,7)=result1';  threshold_005(:,:,7)=result2';
threshold_008(:,:,7)=result3';  threshold_010(:,:,7)=result4';
load php18.mat;     threshold_003(:,:,8)=result1';  threshold_005(:,:,8)=result2';
threshold_008(:,:,8)=result3';  threshold_010(:,:,8)=result4';
load php19.mat;     threshold_003(:,:,9)=result1';  threshold_005(:,:,9)=result2';
threshold_008(:,:,9)=result3';  threshold_010(:,:,9)=result4';
load php110.mat;    threshold_003(:,:,10)=result1'; threshold_005(:,:,10)=result2';
threshold_008(:,:,10)=result3'; threshold_010(:,:,10)=result4';
load php111.mat;    threshold_003(:,:,11)=result1'; threshold_005(:,:,11)=result2';
threshold_008(:,:,11)=result3'; threshold_010(:,:,11)=result4';
load php112.mat;    threshold_003(:,:,12)=result1'; threshold_005(:,:,12)=result2';
threshold_008(:,:,12)=result3'; threshold_010(:,:,12)=result4';
load php113.mat;    threshold_003(:,:,13)=result1'; threshold_005(:,:,13)=result2';
threshold_008(:,:,13)=result3'; threshold_010(:,:,13)=result4';
load php114.mat;    threshold_003(:,:,14)=result1'; threshold_005(:,:,14)=result2';
threshold_008(:,:,14)=result3'; threshold_010(:,:,14)=result4';
load php115.mat;    threshold_003(:,:,15)=result1'; threshold_005(:,:,15)=result2';
threshold_008(:,:,15)=result3'; threshold_010(:,:,15)=result4';
load php116.mat;    threshold_003(:,:,16)=result1'; threshold_005(:,:,16)=result2';
threshold_008(:,:,16)=result3'; threshold_010(:,:,16)=result4';
load php117.mat;    threshold_003(:,:,17)=result1'; threshold_005(:,:,17)=result2';
threshold_008(:,:,17)=result3'; threshold_010(:,:,17)=result4';
load php118.mat;    threshold_003(:,:,18)=result1'; threshold_005(:,:,18)=result2';
threshold_008(:,:,18)=result3'; threshold_010(:,:,18)=result4';
load php119.mat;    threshold_003(:,:,19)=result1'; threshold_005(:,:,19)=result2';
threshold_008(:,:,19)=result3'; threshold_010(:,:,19)=result4';
load php120.mat;    threshold_003(:,:,20)=result1'; threshold_005(:,:,20)=result2';
threshold_008(:,:,20)=result3'; threshold_010(:,:,20)=result4';
load php121.mat;    threshold_003(:,:,21)=result1'; threshold_005(:,:,21)=result2';
threshold_008(:,:,21)=result3'; threshold_010(:,:,21)=result4';
load php122.mat;    threshold_003(:,:,22)=result1'; threshold_005(:,:,22)=result2';
threshold_008(:,:,22)=result3'; threshold_010(:,:,22)=result4';
load php123.mat;    threshold_003(:,:,23)=result1'; threshold_005(:,:,23)=result2';
threshold_008(:,:,23)=result3'; threshold_010(:,:,23)=result4';
load php124.mat;    threshold_003(:,:,24)=result1'; threshold_005(:,:,24)=result2';
threshold_008(:,:,24)=result3'; threshold_010(:,:,24)=result4';
load php125.mat;    threshold_003(:,:,25)=result1'; threshold_005(:,:,25)=result2';
threshold_008(:,:,25)=result3'; threshold_010(:,:,25)=result4';
load php126.mat;    threshold_003(:,:,26)=result1'; threshold_005(:,:,26)=result2';
threshold_008(:,:,26)=result3'; threshold_010(:,:,26)=result4';
load php127.mat;    threshold_003(:,:,27)=result1'; threshold_005(:,:,27)=result2';
threshold_008(:,:,27)=result3'; threshold_010(:,:,27)=result4';
load php128.mat;    threshold_003(:,:,28)=result1'; threshold_005(:,:,28)=result2';
threshold_008(:,:,28)=result3'; threshold_010(:,:,28)=result4';
load php129.mat;    threshold_003(:,:,29)=result1'; threshold_005(:,:,29)=result2';
threshold_008(:,:,29)=result3'; threshold_010(:,:,29)=result4';
```

```
load php130.mat;    threshold_003(:,:,30)=result1'; threshold_005(:,:,30)=result2';
threshold_008(:,:,30)=result3'; threshold_010(:,:,30)=result4';
load php131.mat;    threshold_003(:,:,31)=result1'; threshold_005(:,:,31)=result2';
threshold_008(:,:,31)=result3'; threshold_010(:,:,31)=result4';
load php132.mat;    threshold_003(:,:,32)=result1'; threshold_005(:,:,32)=result2';
threshold_008(:,:,32)=result3'; threshold_010(:,:,32)=result4';
load php133.mat;    threshold_003(:,:,33)=result1'; threshold_005(:,:,33)=result2';
threshold_008(:,:,33)=result3'; threshold_010(:,:,33)=result4';
load php134.mat;    threshold_003(:,:,34)=result1'; threshold_005(:,:,34)=result2';
threshold_008(:,:,34)=result3'; threshold_010(:,:,34)=result4';
load php135.mat;    threshold_003(:,:,35)=result1'; threshold_005(:,:,35)=result2';
threshold_008(:,:,35)=result3'; threshold_010(:,:,35)=result4';
load php136.mat;    threshold_003(:,:,36)=result1'; threshold_005(:,:,36)=result2';
threshold_008(:,:,36)=result3'; threshold_010(:,:,36)=result4';
load php137.mat;    threshold_003(:,:,37)=result1'; threshold_005(:,:,37)=result2';
threshold_008(:,:,37)=result3'; threshold_010(:,:,37)=result4';
load php138.mat;    threshold_003(:,:,38)=result1'; threshold_005(:,:,38)=result2';
threshold_008(:,:,38)=result3'; threshold_010(:,:,38)=result4';
load php139.mat;    threshold_003(:,:,39)=result1'; threshold_005(:,:,39)=result2';
threshold_008(:,:,39)=result3'; threshold_010(:,:,39)=result4';
load php140.mat;    threshold_003(:,:,40)=result1'; threshold_005(:,:,40)=result2';
threshold_008(:,:,40)=result3'; threshold_010(:,:,40)=result4';

%% Plot 3D: transparent plus inside
figure

        fv_3 = isosurface(flow_space,Apep_space,PI_space,threshold_003);
        fv_5 = isosurface(flow_space,Apep_space,PI_space,threshold_005);
        fv_8 = isosurface(flow_space,Apep_space,PI_space,threshold_008);
        fv_10 = isosurface(flow_space,Apep_space,PI_space,threshold_010);% apep x, flow y, activation z
            if  proinhibitor == 1
                % Ac
                p1 = patch(fv_3,'FaceColor',[0.9 0.9 0.9],'EdgeColor','none'); alpha(0.2)% grey
%                 p1 = patch(fv_5,'FaceColor',[0.9 0.9 0.9],'EdgeColor','none'); alpha(0.2)% grey
                p1 = patch(fv_8,'FaceColor',[0.7 0.7 0.7],'EdgeColor','none'); alpha(0.2)% grey
                p3 = patch(fv_10,'FaceColor',[0 0 0],'EdgeColor','none'); % black
            elseif proinhibitor == 2
                %ZGly
                p1 = patch(fv_3,'FaceColor',[1  1 0.4],'EdgeColor','none'); alpha(0.2)% yellow
%                 p1 = patch(fv_5,'FaceColor',[1  1 0.4],'EdgeColor','none'); alpha(0.1)%
                p1 = patch(fv_8,'FaceColor',[1  1 0.4],'EdgeColor','none'); alpha(0.2)%
                p3 = patch(fv_10,'FaceColor',[0.55 0.4 0],'EdgeColor','none');

            elseif proinhibitor == 3
                %Asp
                p1 = patch(fv_3,'FaceColor',[1 .6 0],'EdgeColor','none'); alpha(0.2)% orange
%                 p1 = patch(fv_5,'FaceColor',[1 .6 0],'EdgeColor','none'); alpha(0.1)%
                p1 = patch(fv_8,'FaceColor',[1 .6 0],'EdgeColor','none'); alpha(0.2)%
                p3 = patch(fv_10,'FaceColor',[1 0.5 0],'EdgeColor','none'); %
            elseif proinhibitor == 4
                %DEG
                p1 = patch(fv_3,'FaceColor',[0 .65 .65],'EdgeColor','none'); alpha(0.1)% cyan
%                 p1 = patch(fv_5,'FaceColor',[0 1 1],'EdgeColor','none'); alpha(0.2)%
                p1 = patch(fv_8,'FaceColor',[0 .65 .65],'EdgeColor','none'); alpha(0.1)%
                p3 = patch(fv_10,'FaceColor',[0 .65 .65],'EdgeColor','none');
            end
        view(3); grid on;
                    % Settings labels
%                     title(  ['Oscillating regime' num2str(proinhibitor) '.mat'],...
%                     'FontWeight','bold','FontSize',16,'FontName','Calibri','FontAngle','italic');
%                     xlabel('flow rate (\muL h^-^1)','FontWeight','bold','FontSize',16, 'FontName','Calibri');
%                     ylabel('Apep rate (U mL^-^1)','FontWeight','bold','FontSize',16, 'FontName','Calibri');
%                     zlabel(['[' num2str(proinhibitor) ']_0 (mM)'],'FontWeight','bold',
'FontSize',16,'FontName','Calibri');
                    axis([0 150 0 0.5 0 4]);
                    view([30,90,15])
                    % Settings camera
                        camlight
                        camlight(-80,10)
                        lighting gouraud

end
```

S5 CSTR experiments

S5.1 Fabrication and general setup of PDMS reactors in CSTR experiments

The setup of a typical continuous stirred tank reactor (CSTR) experiment is depicted in Figure S5.1. The CSTR has a volume of 250 µL, is made of polydimethylsiloxane (PDMS), uses built-in temperature control, and is fed by four separate inlets to supply for trypsinogen, trypsin, aminopeptidase, and pro-inhibitor. The concentration of Tr in the outflow is measured using Tr activity assay reported in experimental section of main text.

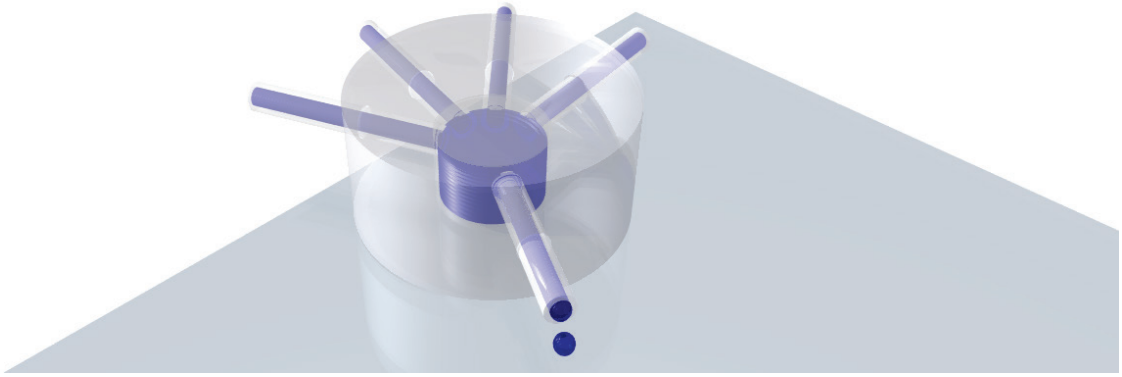


Figure S5.1 *Schematic representation of of PDMS reactors used in CSTR experiments.*

CSTRs are prepared by covering a brass cylinder and a bent copper tube with a mixture of 9 wt% Sylgard® 184 silicone elastomer curing agent crosslinker in Sylgard® 184 silicone elastomer base, which is degassed under reduced pressure. After polymerization of the elastomer mixture at 65 °C for 2 hours PDMS is formed. Then the brass cylinder is removed, leaving a cavity that would serve as the reactor. Holes for the in- and outlets are punched into the PDMS, and the reactor is bonded to a glass surface via oxygen plasma treatment. Then, the reactor is put to 100 °C overnight. Finally, Teflon tubing with appropriate inner diameters (0.56 mm for inlets, 0.38 mm for outlets) is inserted into the punched holes, and silicon tubing is connected to the copper tube to enable a connection to a thermostatic water bath.

S5.2 Experimental conditions flow experiments

Experiments performed in Figure 5 of main text are based on conditions reported below. Figure S5 shows two,*(flow,Ap)*-phase plots for pro-inhibitor **2-4** at initial and newly found $[\mathbf{x}]_0$. In all plots, we have assigned the initial conditions used in the experiments under flow conditions.

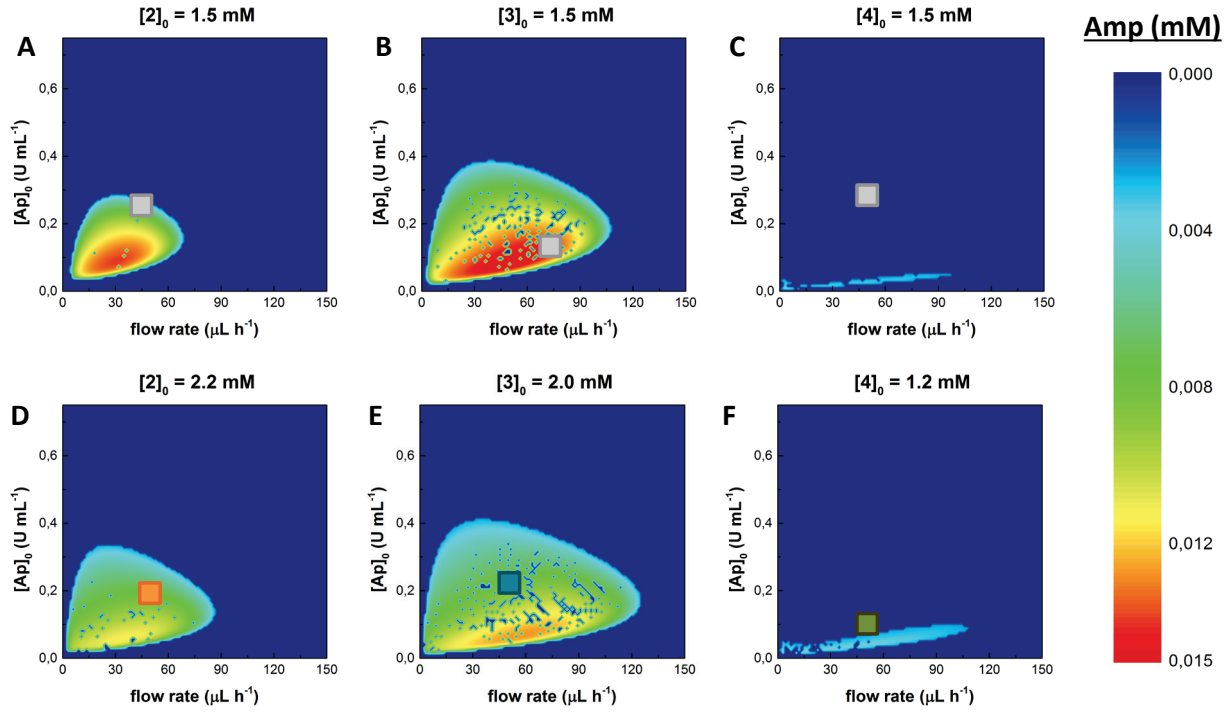


Figure S5.2 *(flow,Ap)-phase plots at different conditions for pro-inhibitors* ***2-4****, with conditions used in experimental flow experiments indicated with squares (a)* ***2*** *in Figures 2a and 5a of the main text, (b)* ***3*** *in Figures 2b and 5b, (c)* ***4*** *in Figures 2c and 5c, (d)* ***2*** *in Figure 5a, (e)* ***3*** *in Figure 5b, (f)* ***4*** *in Figure 5c.*