Supporting information

Novel Phosphonates and Phosphinate esters as warheads for activity-based probes targeting Furin

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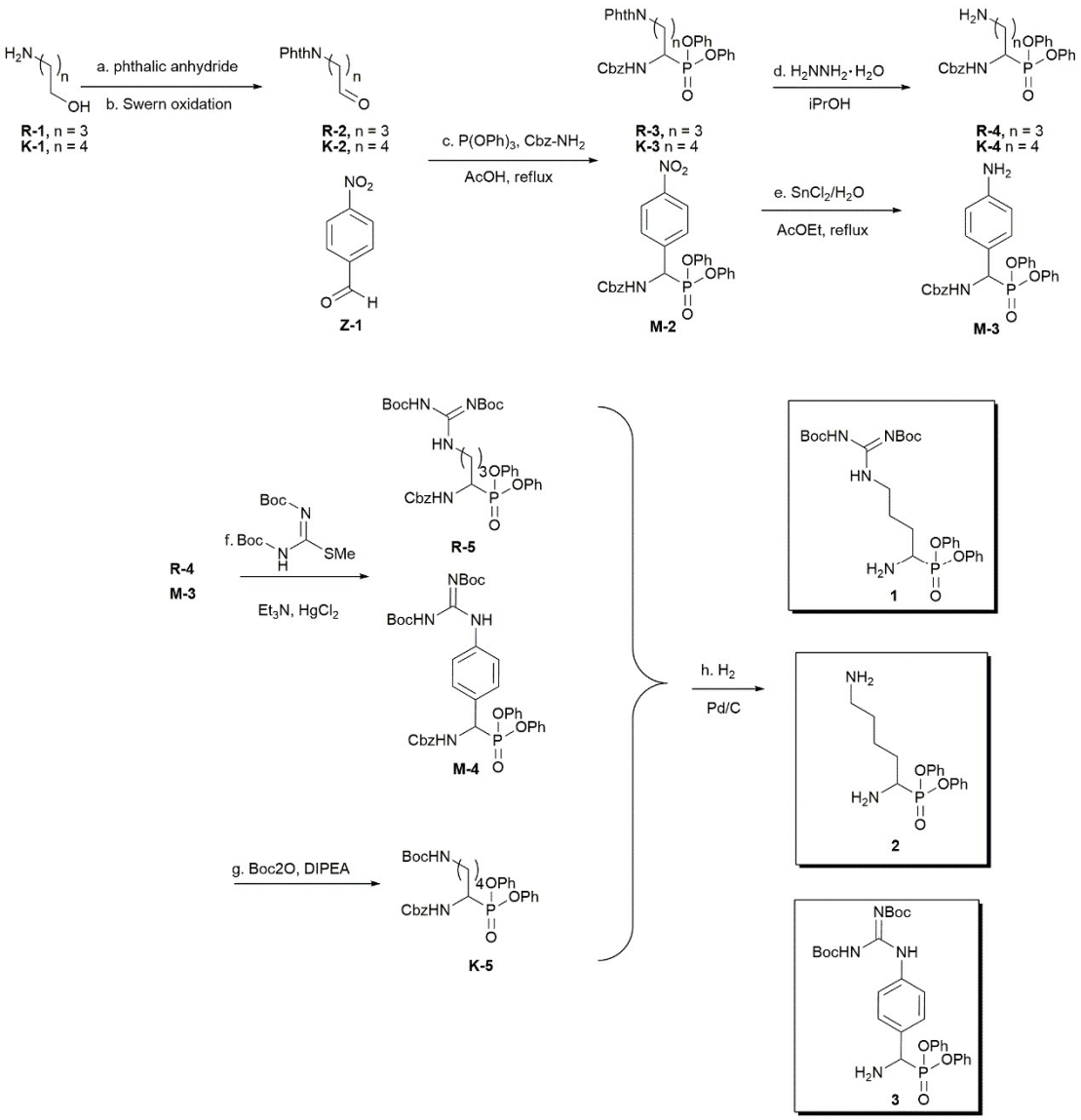
Abbreviations

ABPs	Activity-based probes
AMC	7-amino-4-methyl coumarin
BCA	Bicinchoninic acid assay
Boc	tert-Butyloxycarbonyl
Cbz	benzyloxycarbonyl
CMK	chloromethylketone
DCM	dichloromethane
Dec	Decanoyl
DIC	N, N′-Diisopropylcarbodiimide
DIPEA	N'N-diisopropylethylamine
DMAP	4-Dimethylaminopyridine
DMEM	Dulbecco's modified Eagle medium
DMF	dimethyformamide
DMSO	dimethyl sulfoxide
DPBS	Dulbecco's phosphate buffered saline
DTT	Dithiothreitol
EA	ethyl acetate
ESI	electron spray ionization
Fmoc	Fluorenylmethyloxycarbonyl
FP-Rh	Fluorophosphonate-rhodamine
HATU	(1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b] pyridinium 3-oxide hexafluorophosphate
HBTU	3-[Bis(dimethylamino)methyliumyl]-3H-benzotriazol-1-oxide hexafluorophosphate
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectrometry
LC-MS	liquid chromatography – mass spectrometry

NMP	n-methyl-2-pyrrolidone
NMR	nuclear magnetic resonance
PBS	phosphate buffered saline
PC	proprotein convertases
Ph	phenyl
PhOH	Phenol
Phth	Phthaloyl
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TAMRA	5-Carboxytetramethylrhodamine
TFA	Trifluoroacetic acid
THPTA	Tris(3-hydroxypropyltriazolylmethyl) amine
TLC	thin layer chromatography



Scheme S1. Synthesis of phosphonate building blocks **1**-**3**.

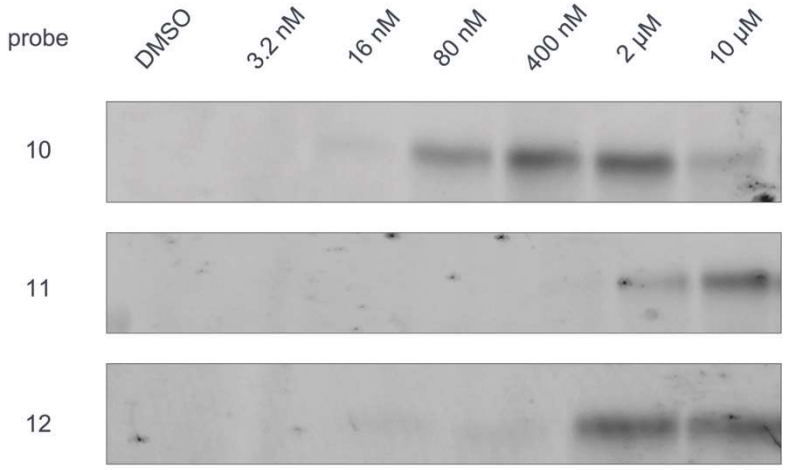
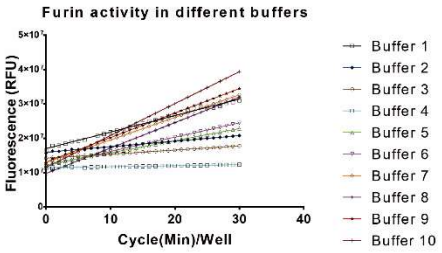
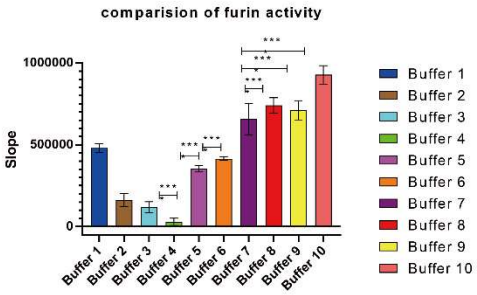


Figure S1. Preliminary labeling of furin revealed that the Arg side chain (probe **10**) was most optimal in comparison with the probe with a P1 side chain of lysine (probe **11**) and p-guanidino-phenylglycine (probe **12**). Probe was added to conditioned medium of furin-overexpressing cells at the indicated concentration and subjected to click chemistry with an azide-fluorophore derivative to enable in gel visualization.

a)



b)



c)

Buffer	Recipe
Buffer 1	20 mM HEPES, 10% glycerol, 0.05%(w/v) DDM, 1 mM $CaCl_2$, pH 7.4
Buffer 2	25 mM HEPES, 150mM NaCl, 1% NP-40, 1 mM $CaCl_2$, pH 7.4
Buffer 3	100 mM HEPES, 10% glycerol, 1 mM $CaCl_2$, pH 7.4
Buffer 4	100 mM HEPES, 1 mM $CaCl_2$, pH 7.4
Buffer 5	100 mM HEPES, 2 mM $CaCl_2$, pH 7.4
Buffer 6	100 mM HEPES, 5 mM $CaCl_2$, pH 7.4
Buffer 7	100 mM HEPES, 1 mM $CaCl_2$, 0.2% Triton X-100, pH 7.4
Buffer 8	100 mM HEPES, 1 mM $CaCl_2$, 0.01% Tween20, pH 7.4
Buffer 9	100 mM HEPES, 1 mM $CaCl_2$, 0.2% Triton X-100, 1mg/ml BSA, pH 7.4
Buffer 10	100 mM HEPES, 1 mM $CaCl_2$, 0.2% Triton X-100, 5mM $MgCl_2$, pH 7.4

Figure S2. Results of a buffer screen to optimize the activity of furin. We found that buffer 10 yields the highest activity, followed by 8, 9 and 7. We observed that 10% glycerol increased activity (see for example buffer 1 or compare buffer 3 with 4). Increasing concentrations of $CaCl_2$ also increased the furin activity (compare buffers 4, 5 and 6). Generally, the addition of a detergent also had a positive influence on the activity (see e.g. the higher slopes of buffers 1 and 7-10). Also the addition of BSA (compare buffer 7 to 9), $MgCl_2$ (compare buffer 7 to 10) increased activity. Overall, we chose buffer 10 (100 mM HEPES, 2 mM $CaCl_2$, 0.2% Triton X-100, 5 mM $MgCl_2$, pH 7.4) as optimal buffer for furin activity.

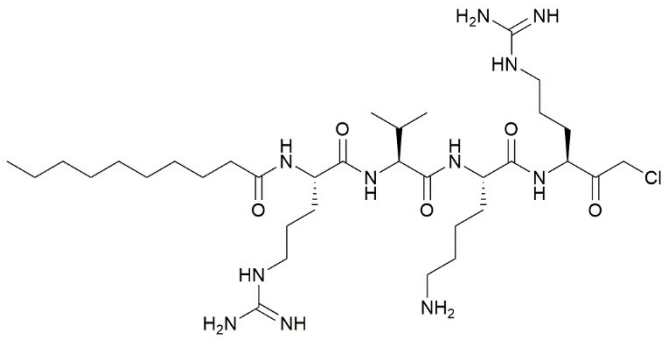


Figure S3. Structure of active-site directed furin inhibitor dec-RVKR-CMK

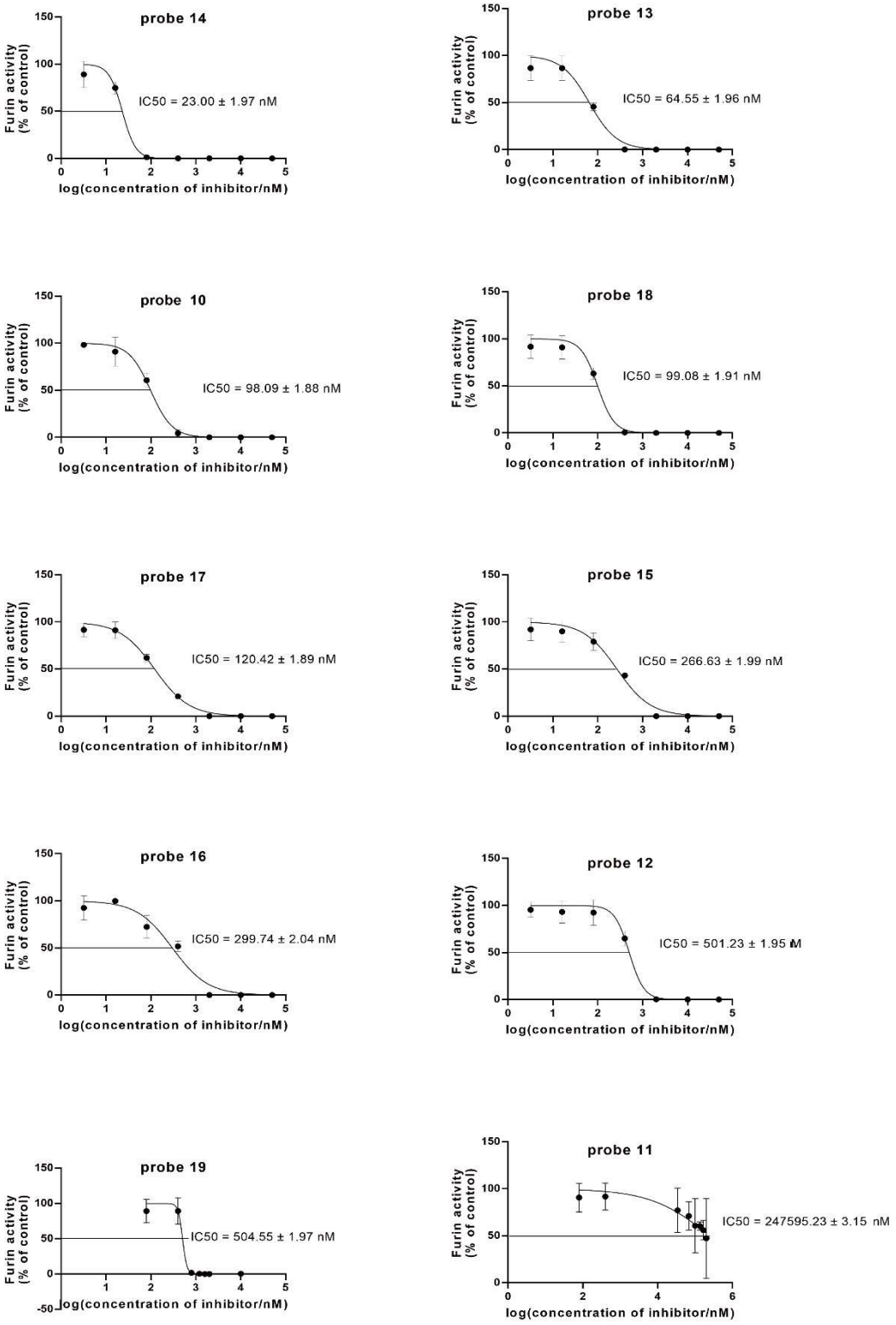


Figure S4. IC_{50} curves for probes **10**-**19** against furin

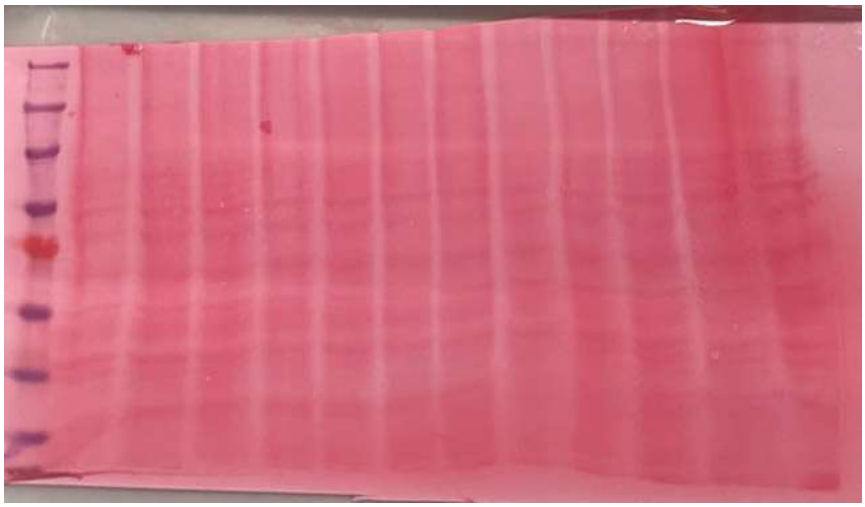


Figure S5. Ponceau loading control of the gel depicted in Figure 2d

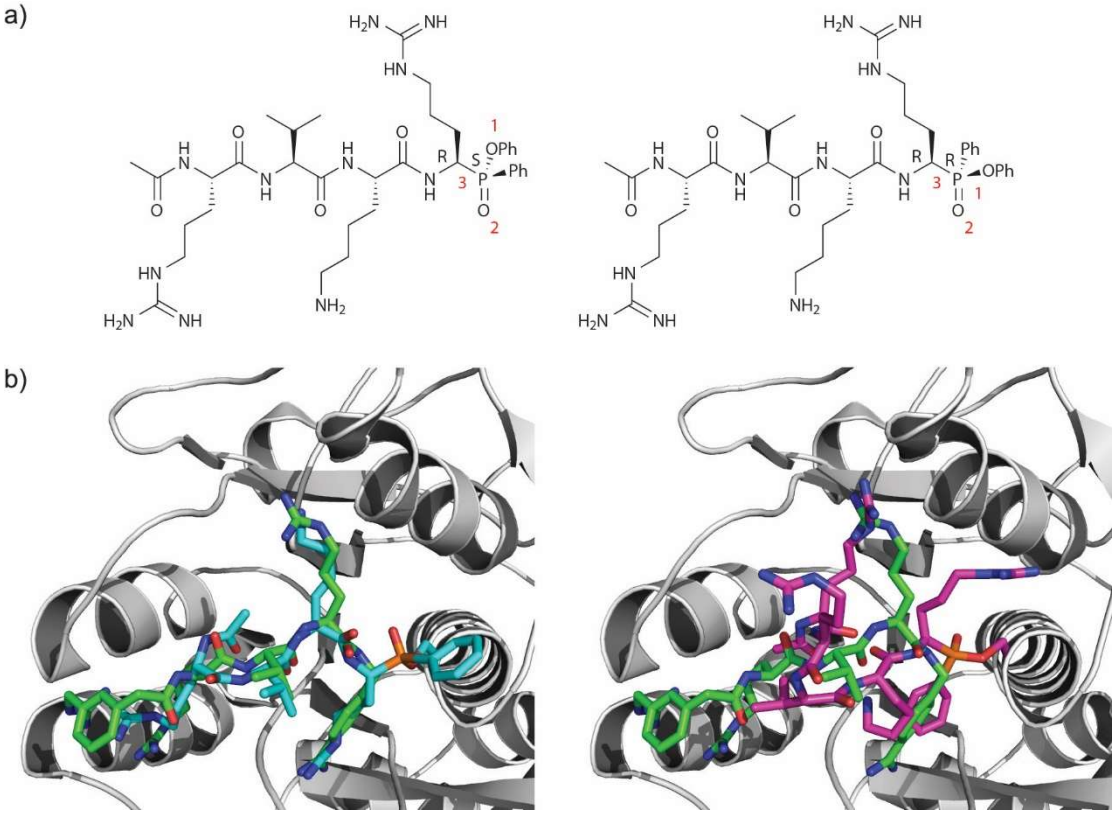


Figure S6. Covalent docking of a phosphinate furin probe. (a) The chiral center at the phosphinate can either be S (left) or R (right). Priorities of the substituents around the phosphor atom are indicated in red. Note that the P=O is considered as a normal single-bonded substituent according to IUPAC rules. Also note that the configuration of the phosphor atom inverts upon S_N2P reaction). (b) Left panel: Phosphinate with the S configuration at the phosphor, covalently docked with attachment to active site serine Ser368 in overlay with the original inhibitor of PDB crystal structure 5JXH. Note the good overlap in the P1, P2 and P3 position. Right panel: same overlay, but then with the covalently docked structure of the other isomer. Note the lack of overlay in the different subsites. The small molecules are shown as stick models and the protein in cartoon mode (in light grey). The original inhibitor is indicated in green, the S isomer in cyan and the R isomer in magenta.

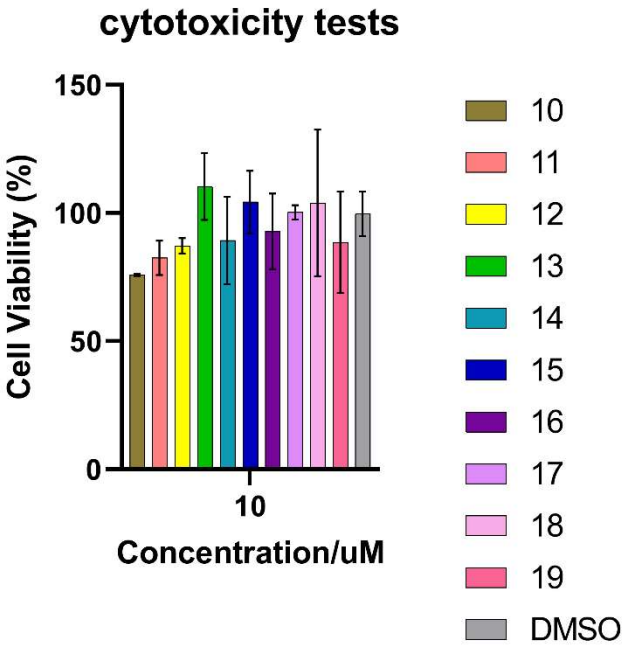


Figure S7. Results of an MTT assay on HEK293T cells with the phosphonate and phosphinate ABPs. Compounds were tested in duplicate at 10 μM for 1h, followed by addition of the MTT reagent.

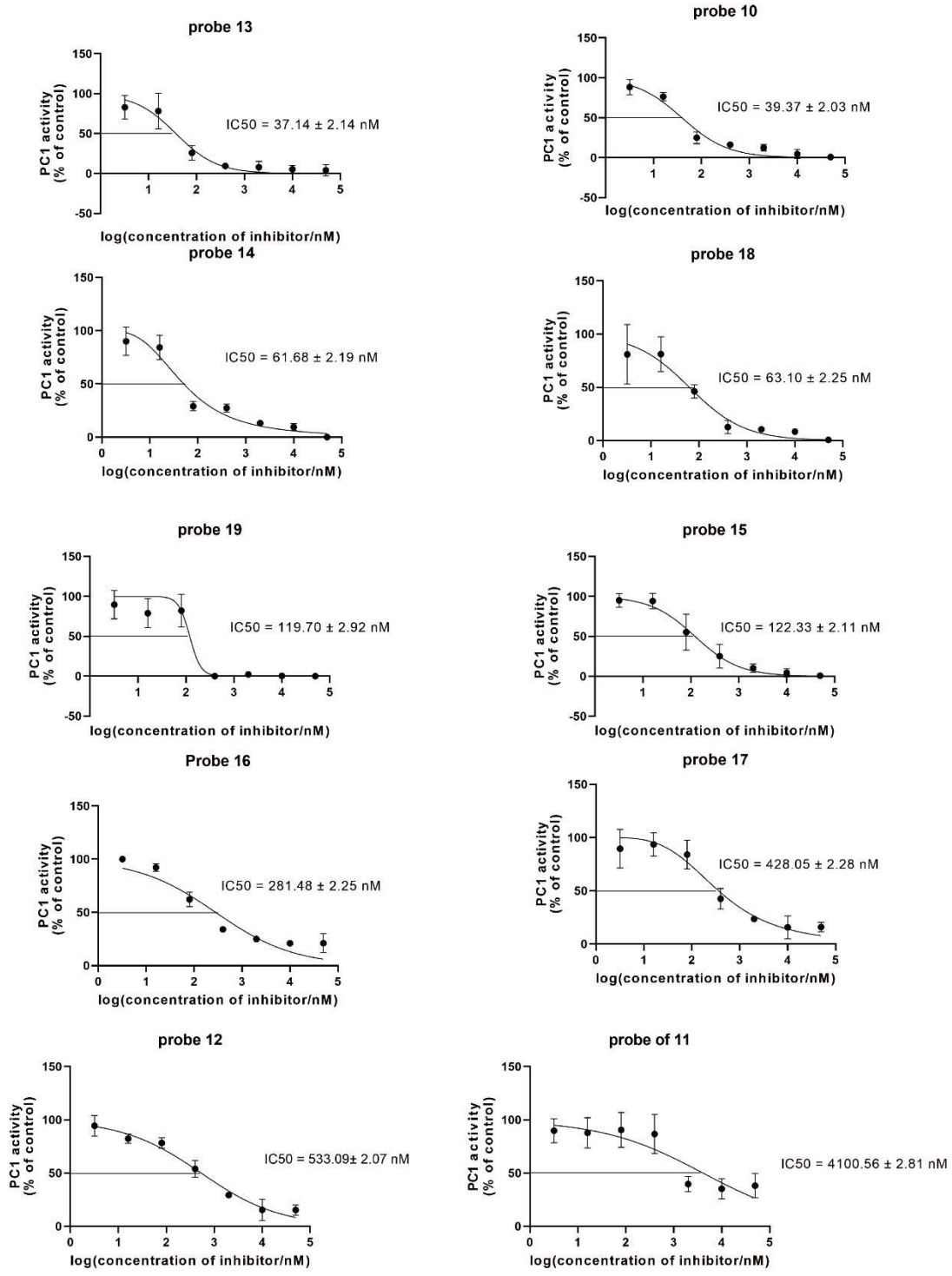


Figure S8. IC_{50}^{App} curves of probes against PC1.

Table S1. Overview of IC_{50}^{App} values (nM) of probes against furin and PC1.

Probe	**IC_{50}^{App} (nM) furin**	**IC_{50}^{App} (nM) PC1**
10	98 ± 1.9	39 ± 2.0
11	>200*10^3	4.1*10^3 ± 2.8
12	501 ± 2.0	533 ± 2.1
13	65 ± 2.0	37 ± 2.1
14	23 ± 2.0	62 ± 2.2
15	266 ± 2.0	122 ± 2.1
16	300 ± 2.0	282 ± 2.3
17	120 ± 1.9	428 ± 2.3
18	99 ± 1.9	63 ± 2.3
19	505 ± 2.0	120 ± 2.9

Synthetic methods

1. General materials and methods

Unless otherwise noted, all the reagents for chemical reactions were purchased from commercial vendors and used without purification. All solvents used for synthesis were of analytical grade or higher. Reaction progress was tested by Thin Layer Chromatography (TLC) on pre-coated 0.20 mm thick ALUGRAM® TLC sheets with fluorescent indicator or by liquid chromatography-mass spectrometry (LC-MS). LC-MS spectra were recorded in a gradient of 5%-90% acetonitrile in water (with 0.1% formic acid) over 30 min on a Prominence Ultra-fast Liquid Chromatography system (Shimadzu) with a Waters X-bridge 2.1 mm C18 column coupled to a MS-2020 single quadrupole mass analyzer (Shimadzu). 230-400 mesh silica (Kieselgel 60) was used to do silica column chromatography. HPLC purification was performed in a gradient of acetonitrile in water (with 0.1% trifluoroacetic acid) over 40 min on a Prominence Ultra-fast Liquid Chromatography system (Shimadzu) with a Waters X-bridge 150 mm C18 prep column. NMR spectra were recorded in deuterated solvent on a Bruker UltraShield 300 MHz spectrometer and were calibrated using tetramethyl silane as an internal reference. Data processing was done using MestReNova. High-resolution mass spectra were acquired on a quadrupole orthogonal acceleration time-of-flight mass spectrometer (Synapt G2 HDMS, Waters, Milford, MA).

2 Synthesis of compounds

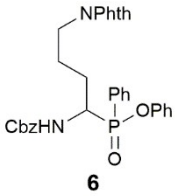
2.1 Phosphonate warhead synthesis

Diphenyl phosphonate esters of Arg (**1**), Lys (**2**), and the p-guanidion-phenylglycine minic (**3**) building blocks were synthesized reported before (Scheme S1).[1-5]

2.2 Arg phosphinate synthesis

The phosphinate synthesis followed a similar procedure as reported by Kahler et al.[6]

Benzyl (4-(1,3-dioxoisoindolin-2-yl)-1-(phenoxy(phenyl)phosphoryl)butyl)carbamate (6)



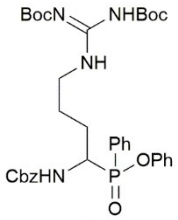
6

In the first step, dichlorophenylphosphine (426.6 µL, 3.14 mmol, 1.3 eq.) was added to a mixture of benzyl carbamate (618.0 mg, 4.08 mmol, 1 eq.) and 4-phthaloylamino-butanal 772.6 mg, 3.55 mmol, 1.13 eq) in dry acetonitrile (5 mL). The mixture was reacted for 14h and full conversion of benzyl carbamate was seen by TLC. The reaction solvent was removed and the residue was dissolved in EA. The mixture was extracted with saturated sodium bicarbonate solution (3x). The combined aqueous phases were adjusted to pH 1 and extracted with EA (3x). Finally, the organic layer was dried by anhydrous magnesium sulfate and concentrated under vacuum, giving intermediate phosphinic acid **5** as an yellow oil, 902.8 mg yield 58.3%, Molecular formula: $C_{26}H_{25}N_2O_6P$. ESI-MS $[M+H]^+$: calculated 493.15; found: 493.15. **5** was used directly in the next step without further purification.

In the second step, DMAP (21.3mg, 0.18 mmol, 0.1eq.), phenol (189.7 mg, 2.02 mmol, 1.1 eq), and phosphinic acid **5** (902.8mg, 1.83 mmol, 1 eq.) were dissolved in 5mL dry toluene. After addition of DIC (1.304 mL, 8.42 mmol, 4 eq.), the mixture was heated to 80 °C for 14h, when TLC of the reaction(EA:PE = 1:1) indicated no further reaction progress of the reaction. The reaction solvent was removed and the residue dissolved in DCM. The organic phase was washed with 1 M $KHSO_4$ (3x), water (3x), saturated sodium bicarbonate (3x) and brine (3x). Finally, the organic layer was dried by anhydrous magnesium sulfate and concentrated under reduced pressure. The crude product was purified using silica column chromatography (PE:EA = 5: 1), and yielded final product **6** (white solid, 871.9 mg, yield 83.6 %). ^{1}H NMR (600 MHz, CDCl3) δ 7.85 (dd, J = 7.7, 4.3 Hz, 2H), 7.82 (dd, J = 5.4, 3.1 Hz, 2H), 7.71 (m, 2H), 7.53 (m, 1H), 7.42 (m, 2H), 7.31 (m, 4H), 7.21 (m, 1H), 7.21 – 7.17 (m, 2H), 7.12 (dd, J = 7.3, 2.1 Hz, 1H), 7.08 – 7.04 (m, 2H), 5.35 (d, J = 8.1 Hz, 1H), 4.48 – 4.34 (m, 1H), 4.24 (s, 2H), 3.84 (m, 2H), 1.79 – 1.69 (m, 2H), 1.25 (m, 2H). 13C NMR (151 MHz, CDCl3) δ 168.22, 156.99, 136.09, 133.93, 133.19, 132.33, 131.97, 129.63, 128.84, 128.76,

128.49, 128.39, 128.14, 128.06, 127.68, 124.63, 123.22, 120.27, 120.24, 67.20, 66.92, 50.83, 50.11, 49.80, 42.05, 37.08, 29.67, 26.01, 25.65, 24.90, 23.47. Molecular formula: $C_{32}H_{29}N_2O_6P$. ESI-MS $[M+H]^+$: calculated 569.18; found: 569.20. HRMS: m/z calculated $[M+H]^+$ 569.1835, found: 569.1844.

phenyl (1-(((benzyloxy)carbonyl)amino)-2-[bis[[(t-butoxy)carbonyl]amino]methylene] aminopropyl])(phenyl)phosphinate (8)



8

6 (871.9 mg, 1.53 mmol, 1 eq.) was dissolved in i-PrOH and warmed to 60 °C. Subsequently, a solution of hydrazine hydrate (223.9 μL, 4.6 mmol, 3eq.) was added to the mixture. The reaction was allowed to stir for 4h at 60 °C during which a white solid appeared. When the reaction was finished as judged by TLC (EA:PE = 1:1), the solid was filtered and the filtrate was evaporated. The crude material was dissolved in $CHCl_3$, washed with brine (3x), dried with anhydrous magnesium sulfate, and then concentrated under reduced pressure. The crude product, compound **7**, was obtained an colourless oil (467.2 mg, yield 69.4 %). Molecular formula: $C_{24}H_{27}N_2O_4P$. ESI-MS $[M+H]^+$: calculated 439.17 found: 439.15. Compound **7** was used directly in the next step without further purification.

Compound **7** (438.46 mg, 1 mmol, 1 eq), 1,3-di-Boc-2-methylisothiourea (304.6 mg, 1.05 mmol, 1.05 eq) and triethylamine (0.31 mL, 2.2 mmol, 2.2 eq) were dissolved in dry DMF (6 mL) at 0 °C and then $HgCl_2$ (299 mg, 1.1 mmol, 1.1 eq) was added. The reaction was finished after 2h as judged by TLC (EA:PE = 2:1). Then the mixture was diluted with EA and filtered. The solution was washed with water and the aqueous phase was back extracted using EA. Then the combined organic phases were filtered, washed with brine (3x), dried by anhydrous magnesium sulfate, and then concentrated under reduced pressure. The crude product was purified using silica column chromatography (PE:EA = 5：1), yielding final product **8** (yellow oil, 461.5 mg, yield 67.8 %).

^{1}H NMR (300 MHz, Chloroform-*d*) δ 11.48 (s, 1H), 8.32 (t, J = 5.6 Hz, 1H), 7.84 (dd, J = 12.0, 7.1 Hz, 2H), 7.59 – 7.49 (m, 1H), 7.42 (m, 2H), 7.35 – 7.24 (m, 4H), 7.21 (m, 2H), 7.09 (dd, J = 8.8, 6.0 Hz, 4H), 5.41 (d, J = 10.6 Hz, 1H), 5.15 – 4.77 (m, 2H), 4.58 – 4.34 (m, 1H), 3.63 – 3.25 (m, 2H), 1.92 – 1.71 (m, 4H), 1.49 (s, 18H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 163.49, 156.23, 155.89, 155.85, 153.24, 136.18, 133.01, 132.49, 132.42, 132.30, 132.23, 129.64, 129.58, 128.85, 128.76, 128.55, 128.46, 128.37, 128.09, 128.03, 127.92, 127.73, 83.09, 79.24, 67.25, 67.10, 50.35, 40.20, 29.66, 28.23, 28.02, 25.99, 25.88, 25.80, 25.53. Molecular formula: $C_{35}H_{45}N_4O_9P$. ESI-MS $[M+H]^+$: calculated 681.30; found: 681.35. HRMS: m/z calculated $[M+H]^+$ 681.3047, found: 681.3062.

2.3 Solid phase chemistry procedures

The loading of the chlorotrityl resin was performed as follows. Fmoc-protected amino acid (3 eq.) and DIPEA (6 eq.) was dissolved in DCM (approximately 10 mL per gram of resin), with a little DMF dissolve all material. Then the mixture was added to chlorotrityl chloride resin (1.6 mmol/g) and shaken for 3h. After that methanol (approximately 0.8 mL per gram of resin) was added to above mixture and shaken for 15 min. Next, the resin was washed with DMF (3x), and the resin loading was determined to be 0.6 mmol/g. Next, the peptide was elongated as follows. Step 1, the Fmoc was removed with 20% piperidine in DMF for 15 min. Step 2, the resin was washed DMF (3x). Next, Fmoc protected amino acid (3 eq.), HBTU (3 eq.) and DIPEA (6 eq.) were shaken for 5 min to be pre-activated in DMF and then added to the resin and shaken for 1h. Completion of the peptide coupling was checked by Kaiser test. Step 1 and 2 were repeated until all building blocks were coupled. The peptides were cleaved from the resin using 1% TFA in DCM and obtained after the solvent was evaporated. The final product was obtained as an oil and was used directly in the next step without further purification.

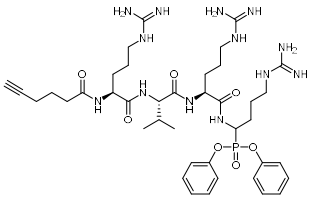
2.4 Peptide coupling with warhead

For phosphonate warheads, A catalytic amount of Pd (10% Pd-C, 20% w/w), and phosphonate were stired in MeOH for 1h under H_2 atmophere in a balloon to remove Cbz-group. For phosphinate warheads, phosphinates were dissolved in 33%

HBr/AcOH and stirred for 30 min. All reactions were analyzed by TLC (EA:PE = 1:1) and solvent was evaporated. The residue was used directly in the next step without further purification.

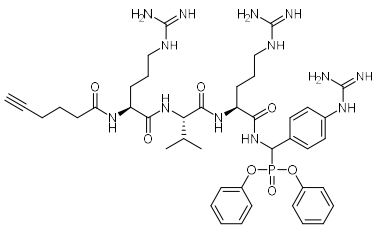
The peptide (1eq.), HATU (1eq.) and collidine (10 eq.) were shaken for 5 min to be pre-actived in NMP (in a volume so that the final peptide concentration was larger than 0.2 M). Next the preactivation mixture was added to the warhead (1.1eq.) and incubated for 5h. When the reaction was finished as tested by LC-MS, the crude solution was dissolved in 95% TFA and reacted for 1h. The solvent was removed and the residue was purified by HPLC.

Diphenyl-((9S,12S,15S)-1-amino-9,15-bis(3-guanidinopropyl)-1-imino-12-isopropyl-8,11,14,17-tetraoxo-2,7,10,13,16-pentaazadocos-21-yn-6-yl) phosphonate (Probe 10)



Probe 10 was synthesized using method 2.4 and purified using HPLC. The product was obtained as a white powder. (11.5 mg, yield = 12.6 %) Molecular formula: $C_{40}H_{62}N_{13}O_7P$. ESI-MS $[M+H]^+$: calculated 868.46; found: 868.50. HRMS: m/z calculated $[M+H]^+$ 868.4705, found: 868.4756.

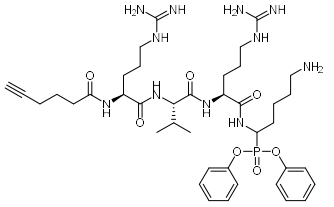
Diphenyl-((4S,7S,10S)-1-(4-guanidinophenyl)-4,10-bis(3-guanidinopropyl)-7-isopropyl-3,6,9,12-tetraoxo-2,5,8,11-tetraazaheptadec-16-yn-1-yl) phosphonate (Probe 11)



Probe 11 was synthesized using method 2.4 and purified using HPLC. The product was obtained as a white powder. (3.3 mg, yield = 12.5 %) Molecular formula:

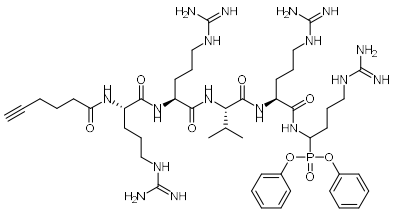
$C_{43}H_{60}N_{13}O_7P$. ESI-MS $[M+H]^+$: calculated 902.45; found: 902.45. HRMS: m/z calculated $[M+H]^+$ 902.4548, found: 902.4555.

Diphenyl-((8S,11S,14S)-1-amino-8,14-bis(3-guanidinopropyl)-11-isopropyl-7,10,13,16-tetraoxo-6,9,12,15-tetraazahenicos-20-yn-5-yl) phosphonate (Probe 12)



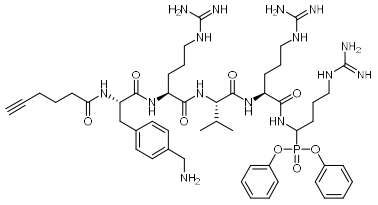
Probe 12 was synthesized using method 2.4 and purified using HPLC. The product was obtained as a white powder. (2.4 mg, yield = 9.8 %) Molecular formula: $C_{40}H_{62}N_{11}O_7P$. ESI-MS $[M+H]^+$: calculated 840.46; found: 840.50. HRMS: m/z calculated $[M+H]^+$ 840.4643, found: 840.4710.

Diphenyl-((9S,12S,15S,18S)-1-amino-9,15,18-tris(3-guanidinopropyl)-1-imino-12-isopropyl-8,11,14,17,20-pentaoxo-2,7,10,13,16,19-hexaazapentacos-24-yn-6-yl) phosphonate (Probe 13)



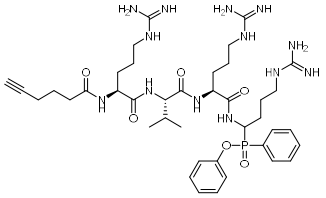
Probe 13 was synthesized using method 2.4 and purified using HPLC. The product was obtained as a white powder. (0.89 mg, yield = 44.5 %) Molecular formula: $C_{46}H_{74}N_{17}O_8P$. ESI-MS $[M+H]^+$: calculated 1024.56; found: 1024.55. HRMS: m/z calculated $[M+H]^+$ 1024.5716, found: 1024.5798.

Diphenyl-((9S,12S,15S,18S)-1-amino-18-(4-(aminomethyl)benzyl)-9,15-bis(3-guanidinopropyl)-1-imino-12-isopropyl-8,11,14,17,20-pentaoxo-2,7,10,13,16,19-hexaazapentacos-24-yn-6-yl) phosphonate (Probe 14)



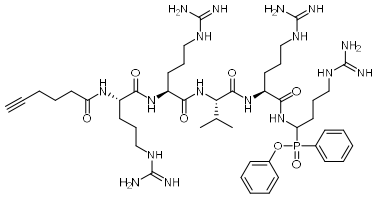
Probe 14 was synthesized using method 2.4 and purified using HPLC. The product was obtained as a white powder. (0.60 mg, yield = 15 %) Molecular formula: $C_{50}H_{74}N_{15}O_8P$. ESI-MS [M+H]+: calculated 1044.56; found: 1044.60. HRMS: m/z calculated $[M+H]^+$ 1044.5654, found: 1044.5730.

Phenyl-((9S,12S,15S)-1-amino-9,15-bis(3-guanidinopropyl)-1-imino-12-isopropyl-8,11,14,17-tetraoxo-2,7,10,13,16-pentaazadocos-21-yn-6-yl)(phenyl) phosphinate (Probe 15)



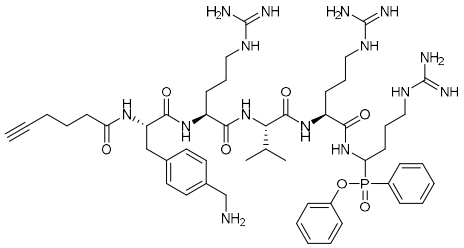
Probe 15 was synthesized using method 2.4 and purified using HPLC. The product was obtained as a white powder. (1.12 mg, yield = 27.1 %) Molecular formula: $C_{50}H_{74}N_{15}O_8P$. ESI-MS $[M+H]^+$: calculated 852.47; found: 852.45. HRMS: m/z calculated $[M+H]^+$ 852.4756, found: 852.4742.

Phenyl-((9S,12S,15S,18S)-1-amino-9,15,18-tris(3-guanidinopropyl)-1-imino-12-isopropyl-8,11,14,17,20-pentaoxo-2,7,10,13,16,19-hexaazapentacos-24-yn-6-yl)(phenyl) phosphinate (Probe 16)



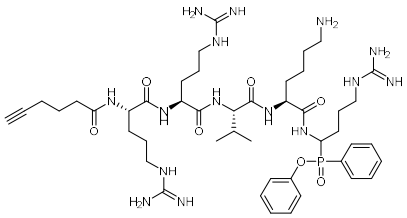
Probe 16 was synthesized using method 2.4 and purified using HPLC. The product was obtained as a white powder. (1.03 mg, yield = 29.3 %) Molecular formula: $C_{46}H_{74}N_{17}O_7P$. ESI-MS $[M+H]^+$: calculated 1008.57; found: 1008.55. HRMS: m/z calculated $[M+H]^+$ 1008.5767, found: 1008.5834.

Phenyl-((9S,12S,15S,18S)-1-amino-18-(4-(aminomethyl)benzyl)-9,15-bis(3-guanidinopropyl)-1-imino-12-isopropyl-8,11,14,17,20-pentaoxo-2,7,10,13,16,19-hexaazapentacos-24-yn-6-yl)(phenyl) phosphinate (Probe 17)



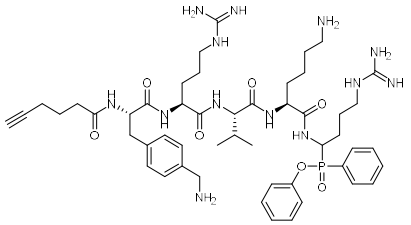
Probe 17 was synthesized using method 2.4 and purified using HPLC. The product was obtained as a white powder. (0.98 mg, yield = 42.8 %) Molecular formula: $C_{50}H_{74}N_{15}O_7P$. ESI-MS $[M+H]^+$: calculated 1028.56; found: 1028.55. HRMS: m/z calculated $[M+H]^+$ 1028.5705, found: 1028.5774.

Phenyl-((9S,12S,15S,18S)-1-amino-9-(4-aminobutyl)-15,18-bis(3-guanidinopropyl)-1-imino-12-isopropyl-8,11,14,17,20-pentaoxo-2,7,10,13,16,19-hexaazapentacos-24-yn-6-yl)(phenyl) phosphinate (Probe 18)



Probe 18 was synthesized using method 2.4 and purified using HPLC. The product was obtained as a white powder. (1.17 mg, yield = 30.0 %) Molecular formula: $C_{46}H_{74}N_{15}O_7P$. ESI-MS $[M+H]^+$: calculated 980.56; found: 980.55. HRMS: m/z calculated $[M+H]^+$ 980.5705, found: 980.5782.

Phenyl-((9S,12S,15S,18S)-1-amino-9-(4-aminobutyl)-18-(4-(aminomethyl)benzyl)-15-(3-guanidinopropyl)-1-imino-12-isopropyl-8,11,14,17,20-pentaoxo-2,7,10,13,16,19-hexaazapentacos-24-yn-6-yl)(phenyl) phosphinate (Probe 19)



Probe 19 was synthesized using method 2.4 and purified using HPLC. The product was obtained as a white powder. (0.78 mg, yield = 29.2 %) Molecular formula: $C_{50}H_{74}N_{13}O_7P$. ESI-MS $[M+H]^+$: calculated 1000.56; found: 1000.55. HRMS: m/z calculated $[M+H]^+$ 1000.5644, found: 1000.5720.

Biochemical methods

3.1 preparation of Furin

The pcDNA3 vector containing a construct for human Furin with a Flag-tag was a gift from John W. M. Creemers. [7] Transient transfection was done according to the following procedure. On day 1, HEK293T cells (2.5 x 10^6 cells) were seeded in 100 mm plates in 10 mL DMEM media (10 % FBS, 1% Streptomycin-Penicillin mixture, 1 % Glutamax); on day 2, the media in the plates was refreshed. 5 µg Furin plasmid was diluted in 1000 µL jetOPTIMUS buffer, and then 5 µL jetOPTIMUS transfection reagent was added. The mixture was shaken and incubated for 10 min, before it was added into the cell medium. On day 3, the medium in the plates was changed to fresh serum-free DMEM media (1% Streptomycin-Penicillin mixture, 1 % Glutamax) and cells were allowed to grow. On day 5, after 48h of serum-free expression, the conditioned media was collected and concentrated using centrifugal filters (30 kDa MWCO Amicon; EMD Millipore). Glycerol was added to the concentrated solution up to 20% (v/v) concentration and the solutions were stored at -80 °C. The concentration of furin in the concentrated, conditioned media was determined to be 48 ng/uL as calculated by band densitometry of a western blot with the conditioned medium and a titration series of purified FLAG-tagged furin (kind gift of J. Creemers, KU Leuven). The conditioned media was used in the following experiments. PC1 was expressed in a similar way using a pcDNA3 vector with a construct for human Flag-tagged PC1 (gift from John W. M. Creemers). After concentration of the conditioned media using centrifugal filters (30

kDa MWCO Amicon; EMD Millipore), PC1 was firstly activated by incubation for 30 min at 37 degrees Celsius in the assay buffer (100 mM NaOAc, 0.1% Triton, 0.1 mg/mL BSA; 5 mM CaCl2, pH 5.5).

3.2 Furin buffer screening

Different furin buffers are used in different papers.[8-12] To make sure to have optimal conditions, 12 buffers were chosen to perform a protease activity test, of which the results are presented in Figure S2. General procedure: furin solution from conditioned media (1uL; total 48.0 ng furin) in 20 µL buffer was incubated for 30 min at 37 °C with 0.2 µL or 2 µL DMSO. Then 80 µL of a 50 µM solution of the corresponding protease substrate (Pyr-Arg-Thr-Lys-Arg-AMC) was added at 37 °C. Fluorescent curves were immediately read for 20 minutes using the SpectraMax iD3 multi-mode plate reader (Molecular Devices) with an excitation wavelength of 370 nm and an emission wavelength of 435 nm. All experiments were done in triplicate at the same time.

3.3 IC_{50} determination for Furin (see Figure S2)

Furin solution (1 uL; total of 48.0 ng furin) in 20 µL buffer was incubated for 30 min at 37 °C with a dilution series of Furin probe **10**-**19** (0-50 µM). 2 µM Furin I inhibitor and an equal volume of DMSO (1% v/v) were used as positive and negative controls. Then 80 µL of a 50 µM solution of the corresponding protease substrate (Pyr-Arg-Thr-Lys-Arg-AMC) in buffer was added at 37 °C. Fluorescent curves were immediately read for 20 minutes using the SpectraMax iD3 multi-mode plate reader (Molecular Devices) with an excitation wavelength of 370 nm and an emission wavelength of 435 nm. Protease activity was calculated from the slope of the linear part of the fluorescence progress curve. The dmso negative control was defined as full activity. IC50 and standard deviation were calculated by prism 9 based on the |log(inhibitor) vs. normalized response with variable slope" and the regression equation: Y=100/(1+10^((LogIC$_{50}$-X)*HillSlope))). All experiments were done in triplicate. For PC1, the IC_{50} values were determined in the same way, but using a different buffer (100 mM NaOAc, 0.1% Triton, 0.1 mg/mL BSA; 5 mM $CaCl_2$, pH 5.5).

3.4 Probes' activity-based test

Furin solution (2.8 uL; 137.2 ng furin) was dissolved in 30 μL Buffer (30 μL 100 mM HEPES, 2 mM $CaCl_2$, 0.2% Triton X-100, 5mM $MgCl_2$, pH 7.4). 2 μM Furin I inhibitor and an equal volume of DMSO (1% v/v) were used as positive and negative controls and were incubated with furin solutin for 1 h at room temperature. Subsequently, the solutions were mixed with different probes with a final concentration of 10 μM. After 1 h incubation at room temperature, the following reagents were sequentially added: TAMRA-azide with final concentration of 50 μM; tris((1-hydroxy-propyl-1H-1,2,3-triazol-4-yl)methyl)amine (THPTA) with final concentration of 50 μM; copper sulfate with final concentration of 1 mM; sodium ascorbate with final concentration of 1 mM. The mixture was reacted for another 1h at room temperature. Next, Laemmli buffer was added to stop the reaction. 20 μL of the mixture was resolved by 7.5 % SDS-PAGE and scanned on a Typhoon FLA 9500.

3.5 Titration of Furin labeling

Furin solution (2.8 uL, 137 ng of furin) in 30 μL buffer (100 mM HEPES, 2 mM $CaCl_2$, 0.2% Triton X-100, 5mM $MgCl_2$, pH 7.4) was incubated at 37 °C with a serial dilution series of furin probe **10**-**19** (0-10 μM). After solution was incubated for 1 h at room temperature, the following reagents were sequentially added: TAMRA-azide with final concentration of 50 μM; tris((1-hydroxy-propyl-1H-1,2,3-triazol-4-yl)methyl)amine (THPTA) with final concentration of 50 μM; copper sulfate with final concentration of 1 mM; sodium ascorbate with final concentration of 1 mM. The mixture was reacted for another 1h at room temperature. Next, 4× Laemmli buffer was added to stop the reaction. 20 μL of the mixture were resolved by 7.5 % SDS-PAGE.and scanned on a Typhoon FLA 9500.

3.6 MTT test

2.5×10^4 HEK293T cells per well were seeded into a 96-well cell culture plate for overnight to adhere. Next, the medium was replaced by 100 μL of culture medium with 10 μM of the ABPs. After incubation for 4h 10 μL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well and incubated for another 4h. Finally, to each well was added 100 μL of MTT solubilization solution and

incubated for another 4h. Subsequently, the absorbance was read at 590 nm and the cell viability was calculated: viability (%) = (absorbance of sample/mean of absorbance of dmso control) × 100.

3.7 Furin labeling in live cells

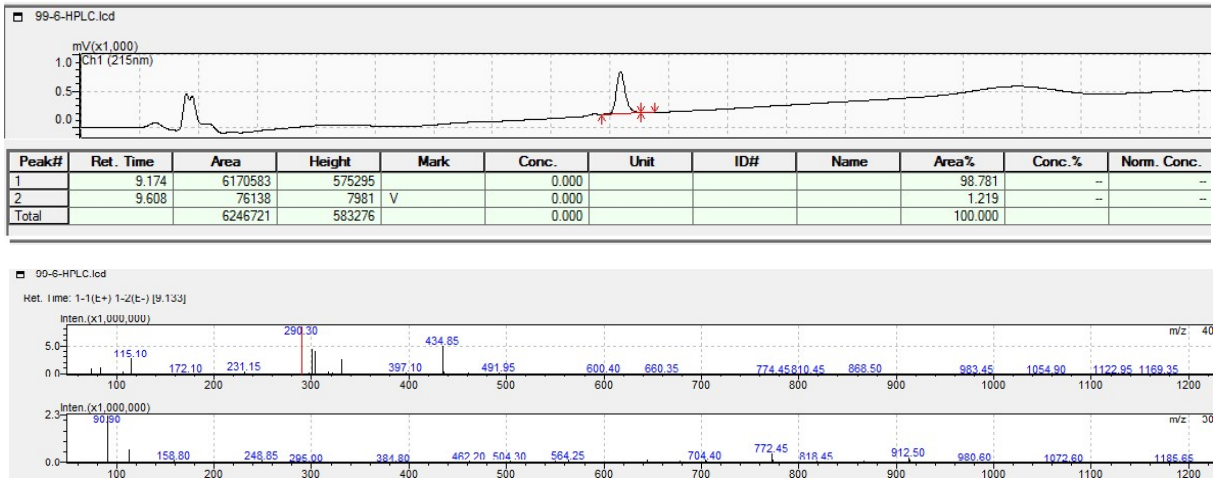
10^5 HEK293T cells were seeded in 24-well plates and incubated overnight to adhere. The cells were transfected with the aforementioned furin plasimid by the jetOPTMUS reagent for 24h. Then the media was removed, and 0.5 mL free-serum DMEM was added. To each sample, 0.5 μL of probes (final concentration 20 μM) was added and incubated with the cells for 2h. The cells were then collected, washed three times with PBS and eventually lysed for 30 minutes on ice using lysis buffer (25 mM HEPES, 150 mM NaCl, pH 7.5 containing 1% NP40). The lysate was centrifuged for 15 minutes at 4 °C, and the supernatant was subjected to 'click'-reaction conditions with TAMRA-azide for one hour as described under section 3.5. The reaction was stopped by addition of sample buffer. The samples were heated to 95 °C for 5 minutes , analyzed by SDS-PAGE using a 12% acrylamide gel and scanned on a Typhoon FLA 9500.

4. Covalent Molecular docking

The atomic coordinates of furin were download from the RCSB Protein Databank (PDB code: 1P8J). Structure of probe **18** was drawn and saved as sdf file by chemdraw 2017. Enzyme and probe **18** were prepared for energy minimization and structure preparation using Molecular Operating Environment (MOE) with forcefield: Amber10-EHT; R-Field 1:80; Cutoff [8,10]. Next, covalent docking was prepared as follows: the chosen warhead reaction phosphinate transesterification, the Refinement option was chosen as Rigid Receptor, and GBVI/WSA dG was chosen as scoring function. The top structure hits were manually inspected and the best pose was selected based on the highest overlap in the different subsites. More details are given in Figure S3.

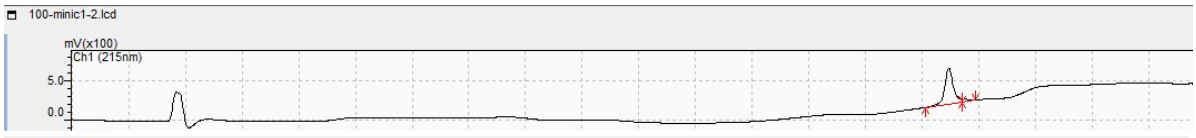
5. Copies of probes' LC-spectra

Probe 10



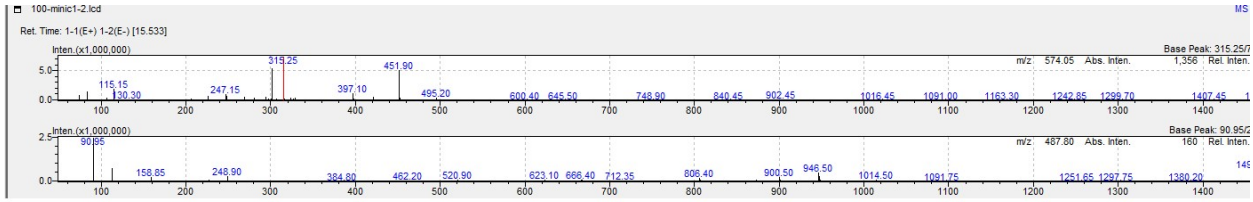
Peak#	Ret. Time	Area	Height	Mark	Conc.	Unit	ID#	Name	Area%	Conc.%	Norm. Conc.
1	9.174	6170583	575295		0.000				98.781	--	--
2	9.608	76138	7981	V	0.000				1.219	--	--
Total		6246721	583276		0.000				100.000		

Probe 11

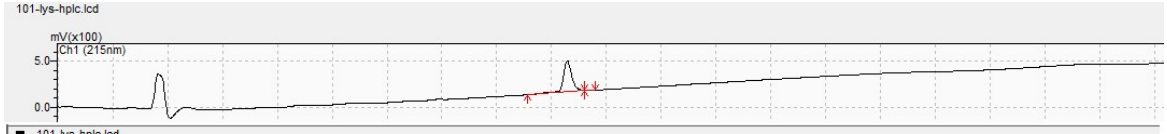


100-minic1-2.lcd

Peak#	Ret. Time	Area	Height	Mark	Conc.	Unit	ID#	Name	Area%	Conc.%	Norm. Conc.
1	15.472	4829066	450461		0.000				95.405	--	--
2	15.766	232594	26931	V	0.000				4.595	--	--
Total		5061660	477392		0.000				100.000		

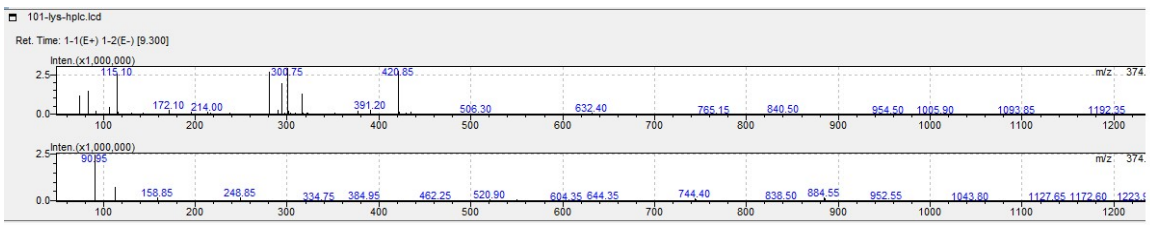


Probe 12

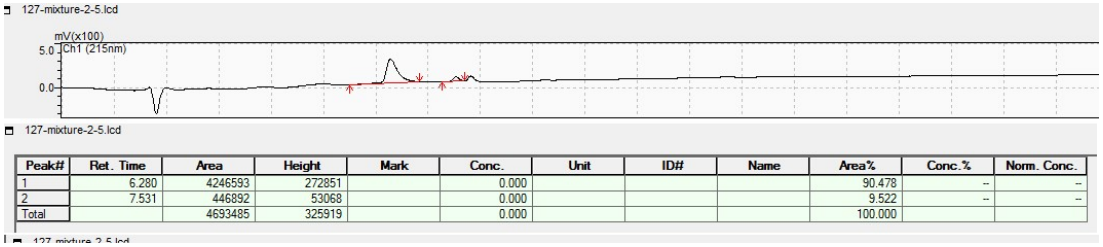


101-lys-hplc.lcd

Peak#	Ret. Time	Area	Height	Mark	Conc.	Unit	ID#	Name	Area%	Conc.%	Norm. Conc.
1	9.289	3702534	336478	M	0.000				98.504	--	--
2	9.704	56237	5148	V	0.000				1.496	--	--
Total		3758771	341626		0.000				100.000		

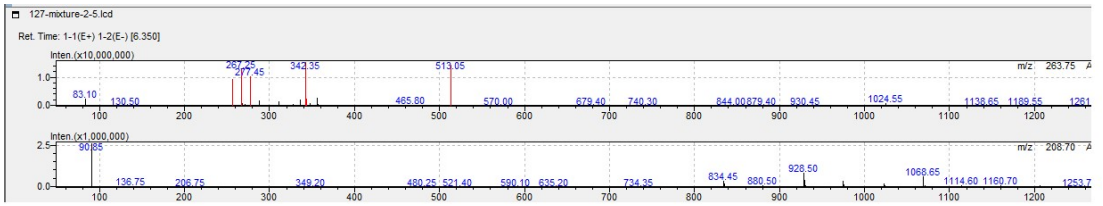


Probe 13

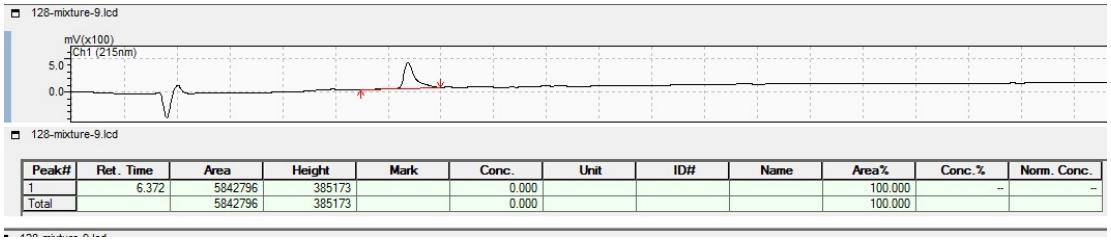


127-mixture-2-5.lcd

Peak#	Ret. Time	Area	Height	Mark	Conc.	Unit	ID#	Name	Area%	Conc.%	Norm. Conc.
1	6.280	4246593	272851		0.000				90.478	--	--
2	7.531	446892	53068		0.000				9.522	--	--
Total		4693485	325919		0.000				100.000		

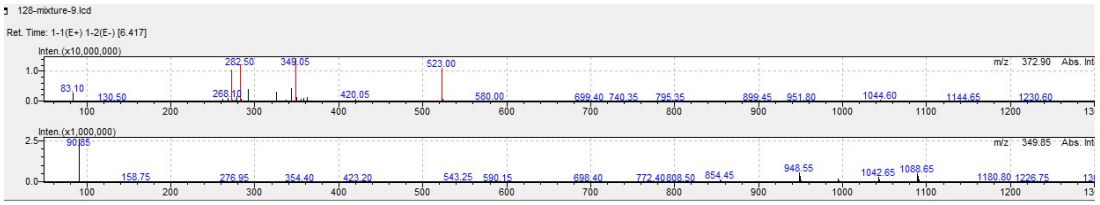


Probe 14

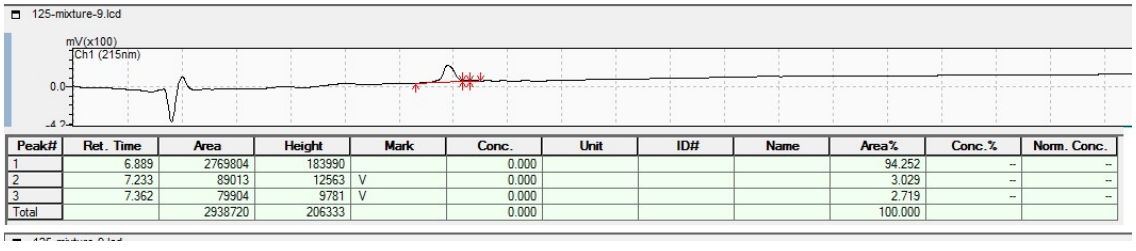


128-mixture-9.lcd

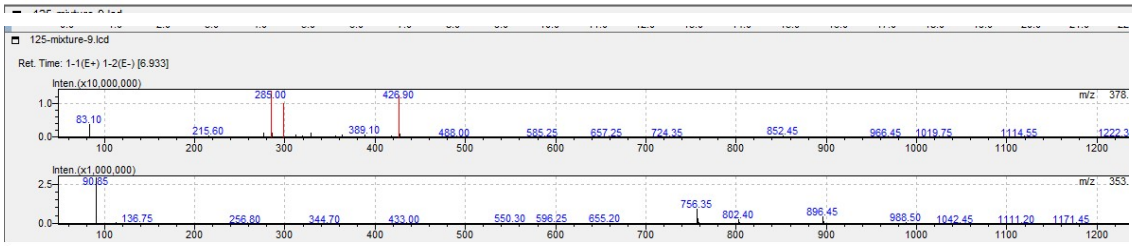
Peak#	Ret. Time	Area	Height	Mark	Conc.	Unit	ID#	Name	Area%	Conc.%	Norm. Conc.
1	6.372	5842796	385173		0.000				100.000	--	--
Total		5842796	385173		0.000				100.000		



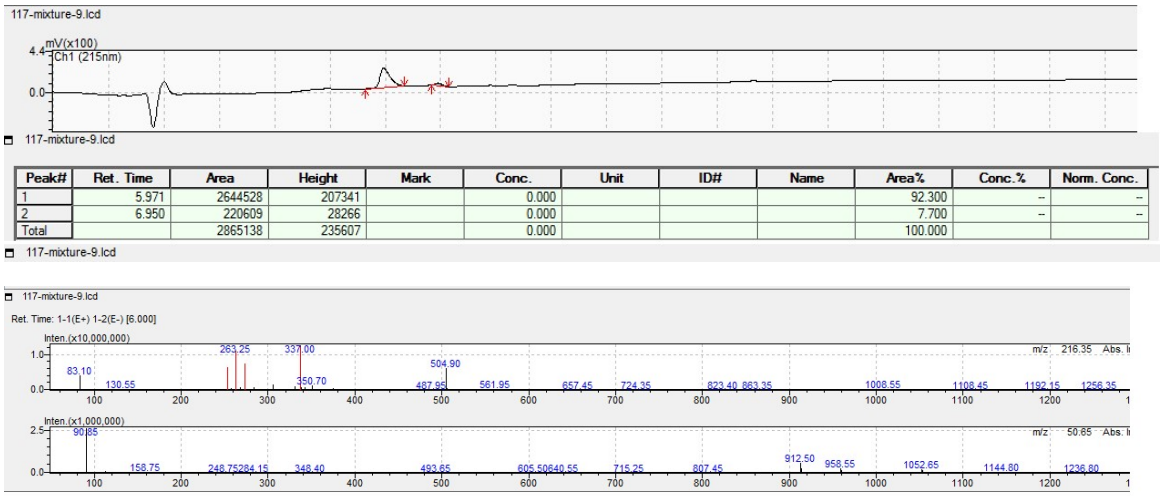
Probe 15



Peak#	Ret. Time	Area	Height	Mark	Conc.	Unit	ID#	Name	Area%	Conc.%	Norm. Conc.
1	6.889	2769804	183990		0.000				94.252	--	--
2	7.233	89013	12563	V	0.000				3.029	--	--
3	7.362	79904	9781	V	0.000				2.719	--	--
Total		2938720	206333		0.000				100.000		

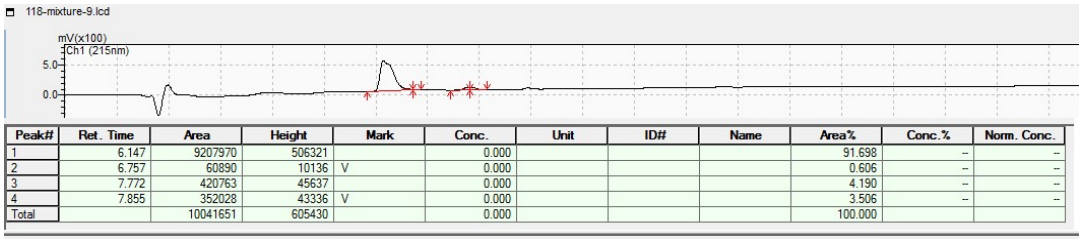


Probe 16

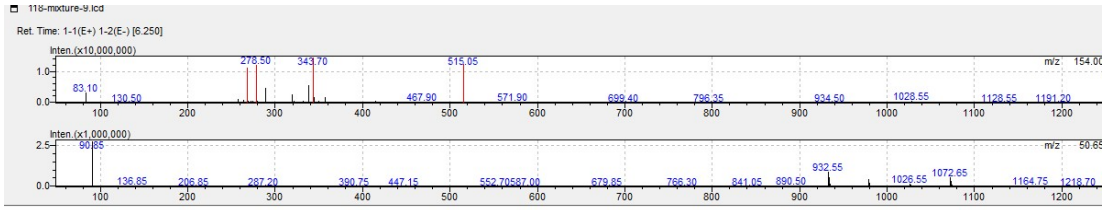


Peak#	Ret. Time	Area	Height	Mark	Conc.	Unit	ID#	Name	Area%	Conc.%	Norm. Conc.
1	5.971	2644528	207341		0.000				92.300	--	--
2	6.950	220609	28266		0.000				7.700	--	--
Total		2865138	235607		0.000				100.000		

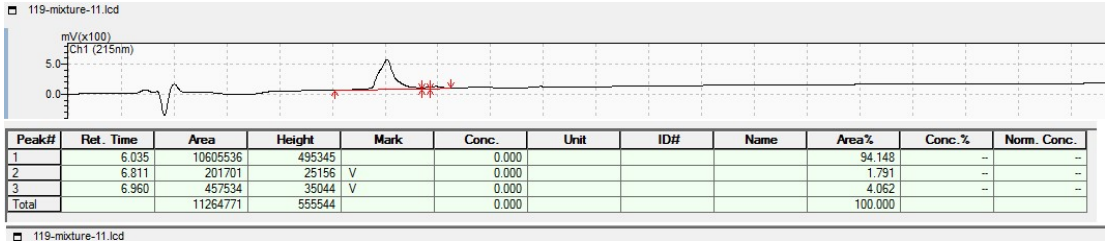
Probe 17



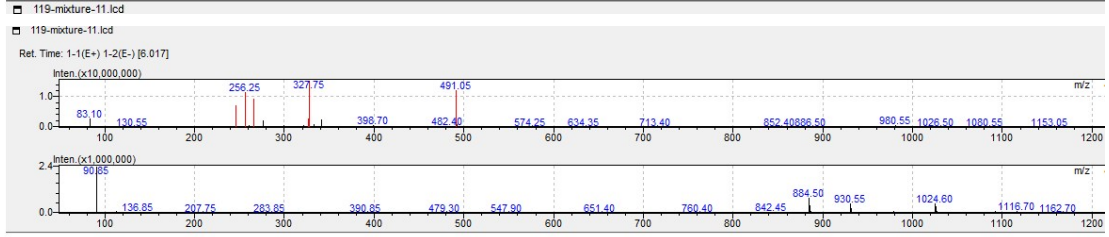
Peak#	Ret. Time	Area	Height	Mark	Conc.	Unit	ID#	Name	Area%	Conc.%	Norm. Conc.
1	6.147	9207970	506321		0.000				91.698	--	--
2	6.757	60890	10136	V	0.000				0.606	--	--
3	7.772	420763	45637		0.000				4.190	--	--
4	7.855	352028	43336	V	0.000				3.506	--	--
Total		10041651	605430		0.000				100.000		



Probe 18



Peak#	Ret. Time	Area	Height	Mark	Conc.	Unit	ID#	Name	Area%	Conc.%	Norm. Conc.
1	6.035	10605536	495345		0.000				94.148	--	--
2	6.811	201701	25156	V	0.000				1.791	--	--
3	6.960	457534	35044	V	0.000				4.062	--	--
Total		11264771	555544		0.000				100.000		



Probe 19

120-mixture-9.lcd

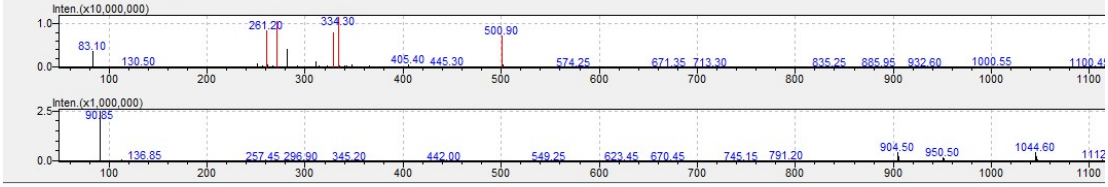
120-mixture-9.lcd

Peak#	Ret. Time	Area	Height	Mark	Conc.	Unit	ID#	Name	Area%	Conc.%	Norm. Conc.
1	5.970	4119880	291369		0.000				98.283	--	--
2	6.825	71975	10702		0.000				1.717	--	--
Total		4191855	302071		0.000				100.000		

120-mixture-9.lcd

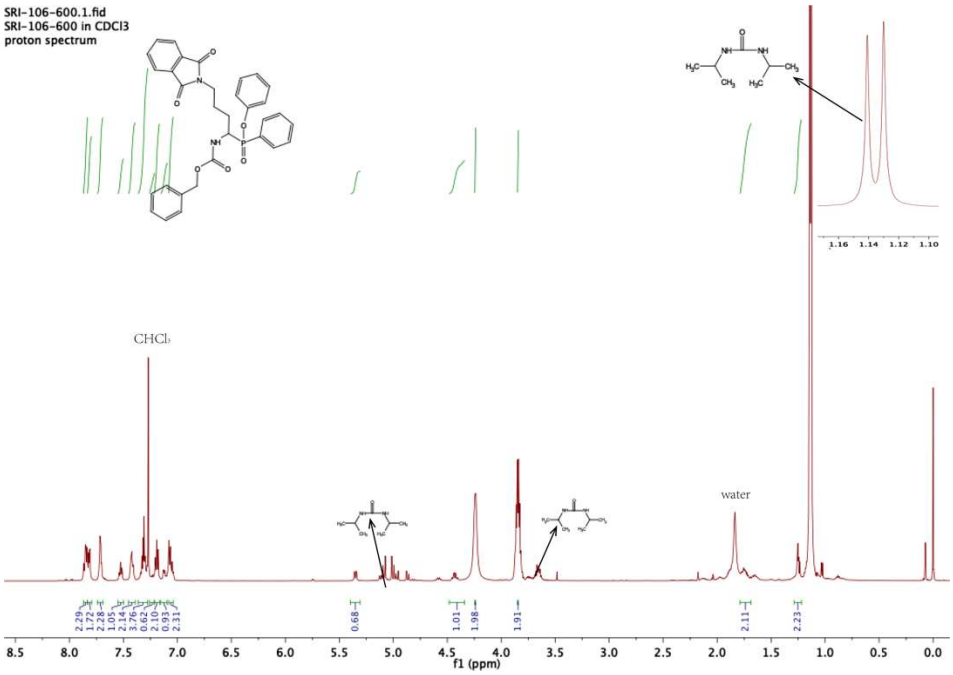
120-mixture-9.lcd

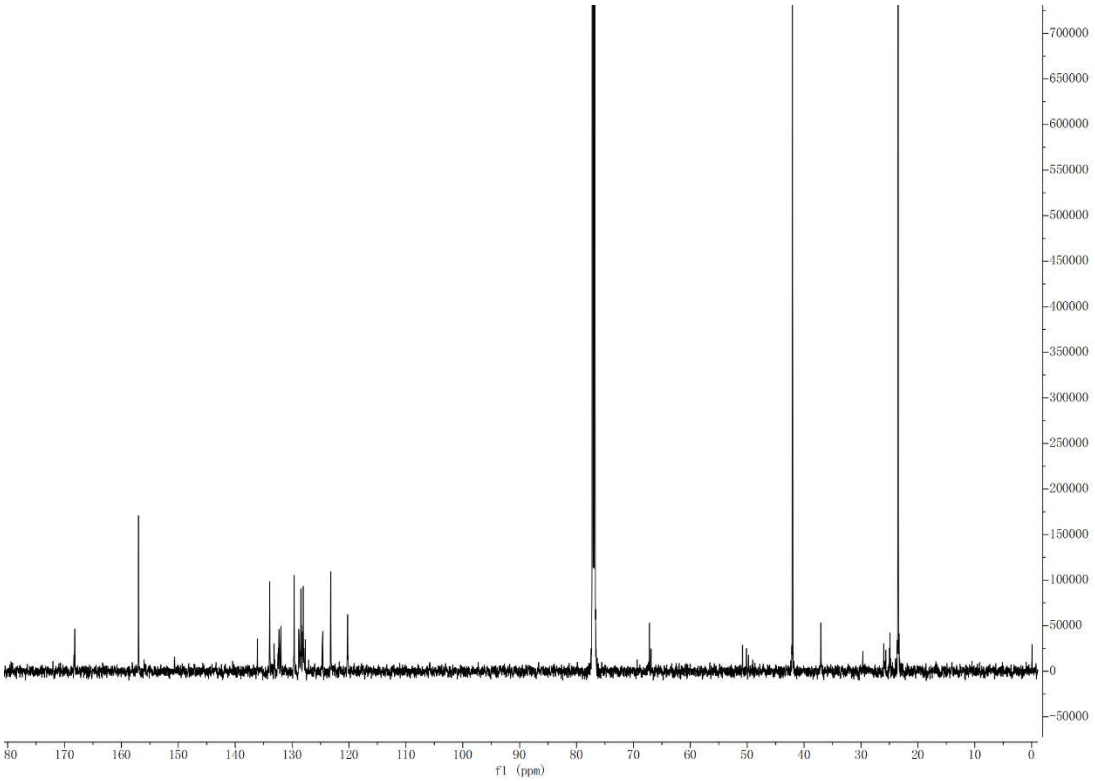
Ret. Time: 1-1(E+) 1-2(E-) [6.017]

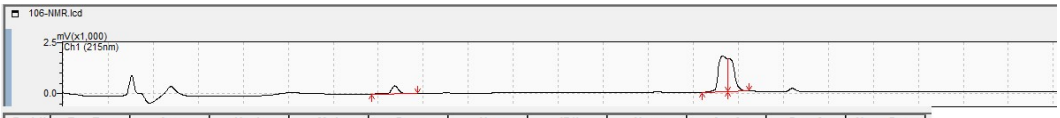


6. NMR Spectra and LC-MS data of compound

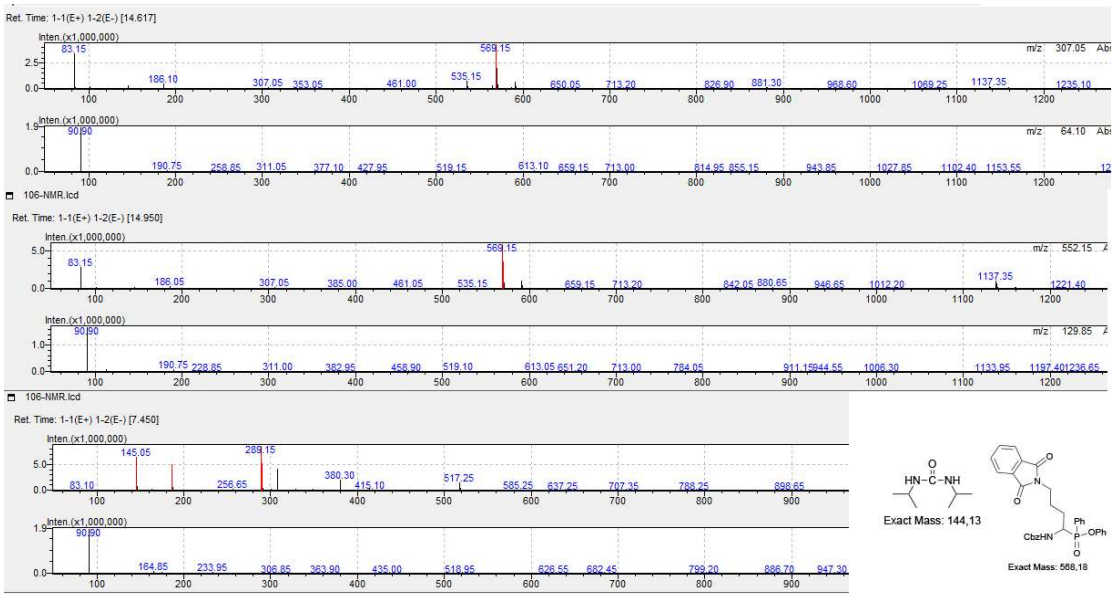
Compound 6, **Rt = 14.65 min. (diastereomer 1); 14.80 min. (diastereomer 2).**



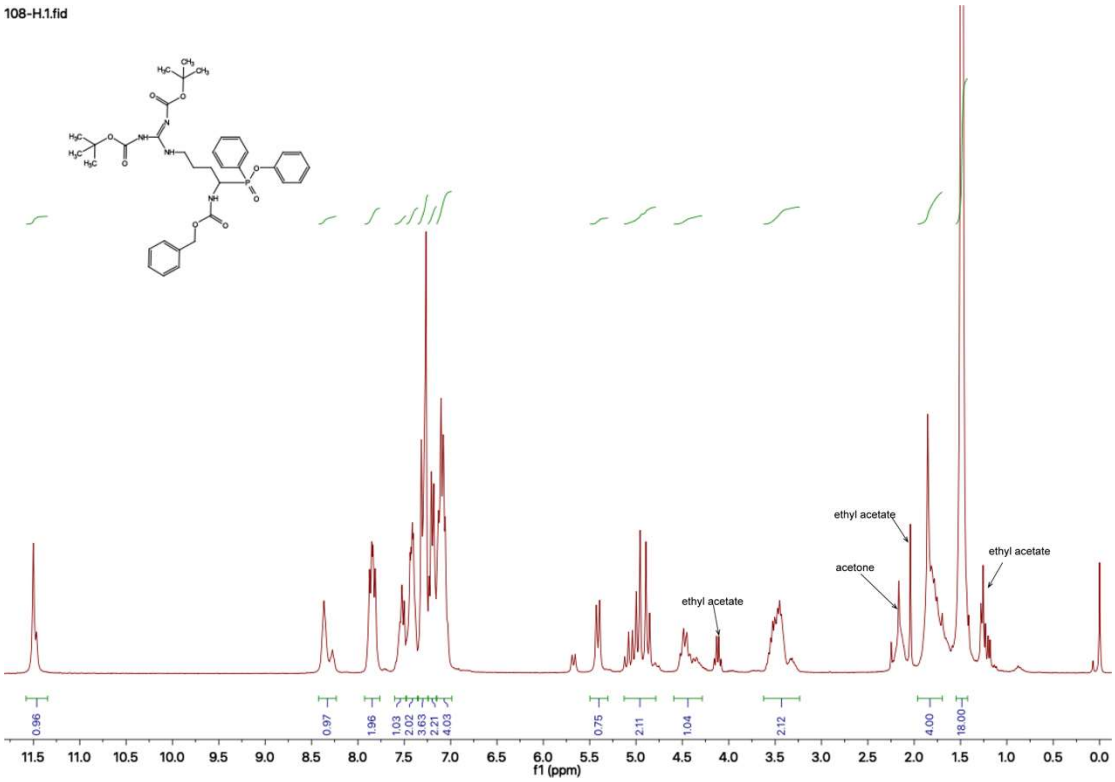




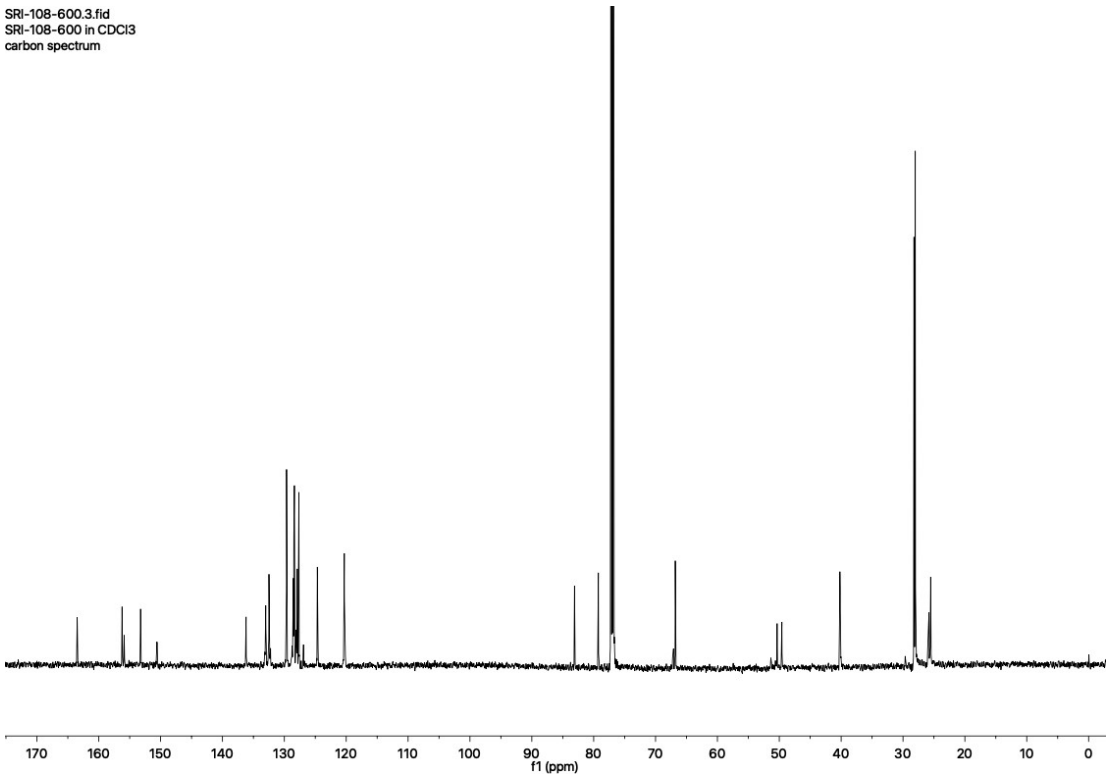
Peak#	Ret. Time	Area	Height	Mark	Conc.	Unit	ID#	Name	Area%	Conc.%	Norm. Conc.
1	7.366	4090574	385136		0.000				9.303	--	--
2	14.654	23590686	1755397		0.000				53.648	--	--
3	14.798	16291519	1604792	V	0.000				37.049	--	--
Total		43972779	3745325		0.000				100.000		

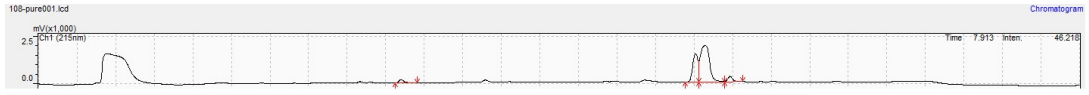


Compound 8, Rt = 17.04 min. (diastereomer 1); 17.29 min. (diastereomer 2).

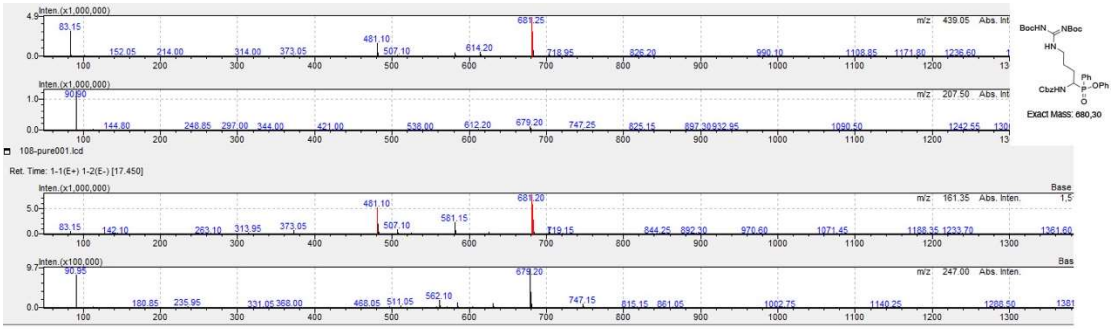


SRI-108-600.3.fid
SRI-108-600 in CDCl3
carbon spectrum





Peak#	Ret. Time	Area	Height	Mark	Conc.	Unit	ID#	Name	Area%	Conc.%	Norm. Conc.
1	9.399	1315354	156721		0.000				2.688	--	--
2	17.040	13681289	1481026		0.000				27.956	--	--
3	17.290	31514183	1903229	V	0.000				64.395	--	--
4	17.934	2427800	281501	V	0.000				4.961	--	--
Total		48938627	3822477		0.000				100.000		



7. Supplementary references

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