Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry

Supporting Information

Rational design of a Cyclin A fluorescent peptide sensor

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General peptide synthesis procedures

All peptide synthesis reagents and amino acid derivatives were purchased from *GL Biochem* (Shanghai) and *NovaBiochem*; amino acids were purchased as protected Fmoc amino acids with the standard side chain protecting scheme: Fmoc-Ala-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Phe-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Gly-OH, Fmoc-Ile-OH, and Fmoc-Asp(OtBu)-OH, Fmoc-His(Trt)-OH, Fmoc-Ser(tBu)-OH except for the orthogonally protected Fmoc-Dap(Alloc)-OH which was purchased from *Bachem* (Cat.#: B−2845). C-terminal amide peptides were synthesized on a 0.05 scale using a 0.21 mmol/g loading Fmoc−PAL−PEG−PS resin from *Applied Biosystems*. 4-Chloro-7-nitrobenzofurazan was purchased from *Aldrich* (Cat.#: 163260-1G) All other chemicals were purchased from *Aldrich* or *Fluka*. All solvents were dry and synthesis grade, except DMF for peptide synthesis.

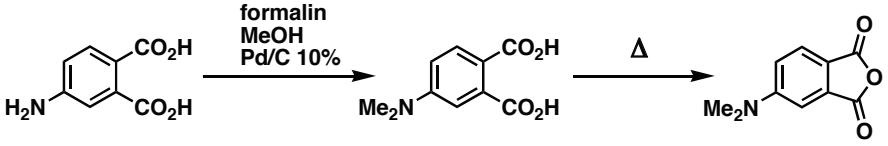
Peptides were synthesized using a *PS3* automatic peptide synthesizer from *Protein Tecnologies*. The amino acids were coupled in 4-fold excess using *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino) methylene]-*N*-methylmethanaminium hexafluorophosphate (HBTU) as activating agent. *N,N*-dimethylaminopthalimide fluorophore was coupled manually in 4-fold excess using a mixture of *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-N-methylmethanaminium (HBTU) and 1-hidroxybenzotriazole (HOBt) as activating agents. Cl-NBD was coupled manually in 10-fold excess. Each amino acid was activated for 30 seconds in DMF before being added onto the resin. Peptide bond-forming couplings were conducted for 30 min to 45min. The deprotection of the temporal Fmoc protecting group was performed by treating the resin with 20% piperidine in DMF solution for 10 min. The final peptides were cleaved from the resin, and side-chain protecting groups were simultaneously removed using a standard TFA cleavage cocktail as outlined below.

High-Performance Liquid Chromatography (HPLC) was performed using an *Agilent 1100* series Liquid Chromatograph Mass Spectrometer system. Analytical HPLC was run using a *Zorbax Eclipse XDB*-C_8 (5 µm) 4.6 × 150 mm analytical column from *Agilent*. The purification of the peptides was performed on a *Jupiter Proteo 90A* (4 µm), 10 × 250 mm reverse-phase column from *Phenomenex*. The standard gradient used for analytical and preparative HPLC was 95:15 to 5:95 over 30 min (water/acetonitrile, 0.1% TFA). Electrospray Ionization Mass Spectrometry (ESI/MS) was performed with an *Agilent 1100* Series LC/MSD VL G1956A model in positive scan mode using direct injection of the purified peptide solution.

UV measurements were made in a *Varian Cary 100 Bio* spectrophotometer using a standard *Hellma* semi-micro cuvette. Concentrations were measured using listed extinction coefficients for each chromophore: 6,480 M^{-1} cm^{-1} at 421 nm for 4-DMAP and 17,400 M^{-1} cm^{-1} at 475 nm for NBD.

Fluorescence spectroscopy measurements were made with a *Jobin−Yvon* Fluoromax−3, (DataMax 2.20), coupled to a *Wavelength Electronics* LFI−3751 temperature controller. All measurements were made with a *Hellma* semi-micro cuvette (105.250-QS) at 20 ºC, using the following settings for 4-DMAP and NBD, respectively: excitation wavelength 395 and 478 nm; excitation slit width 5.0 nm, emission slit width 10.0 nm; increment 1.0 nm; integration time 0.20 s. The emission spectra were recorded from 430 to 700 nm for 4-DMAP and 500 to 650 nm for NBD.

Synthesis of 4-N,N-dimethylaminophthalic anhydride [1]



Scheme S1. Synthesis of 4-N,N-dimethylaminophthalic anhydride.

4-aminophthalic acid (500 mg, 2.76 mmol) was dissolved in MeOH (150 mL). Formalin (15 mL, 36% formaldehyde solution) and Pd/C 10% (200 mg) were added, and the resulting solution was stirred at room temperature under a hydrogen atmosphere for 3 hours. The reaction mixture was filtered through celite and concentrated under reduced pressure to give the desired 4-*N*,*N*-dimethylaminophthalic acid as a white solid, which was placed in a sublimator and heated at 140 °C for 10 h under vacuum to give the desired product as a bright yellow solid (429 mg, 81%).

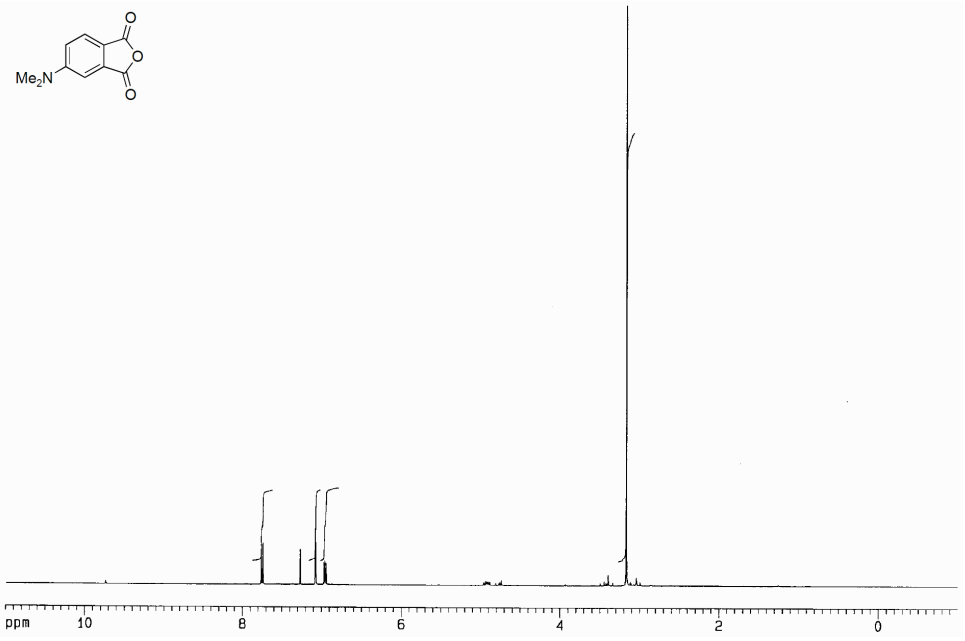
^{1}H NMR (400 MHz, $CDCl_3$, δ): 3.0 (s, 6H), 6.9 (dd, 1H, J_1 = 2.4 Hz, J_2 = 8.7 Hz), 7.07 (s, 1H, J = 2.4 Hz), 7.75 (d, 1H J = 8.6 Hz).

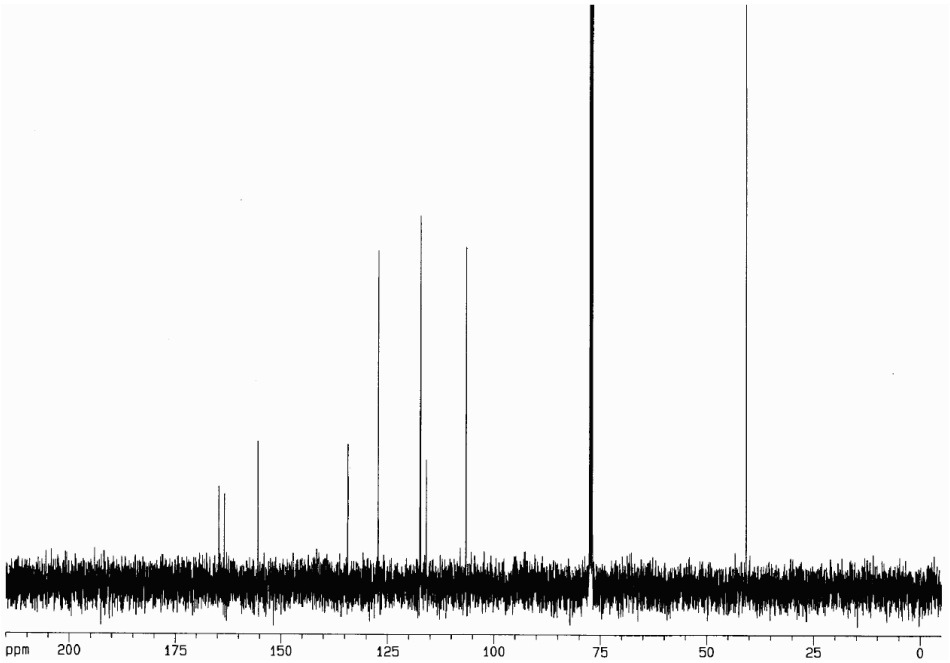
^{13}C NMR (400 MHz, $CDCl_3$, δ): 40.8, 106.6, 115.9, 117.4, 127.1, 134.3, 155.5, 163.4, 164.7.

HRMS-ESI (m/z): [M+H^+] calcd for $C_{10}H_9NO_3$ 192.0655, found, 192.0652.

ESI-MS [M+H^+] calcd for $C_{10}H_9NO_3$ 192.06, found, 192.1. [M+Na^+] calcd for $C_{10}H_9NO_3$ 214.05, found, 214.1.

[1] M. E. Vázquez, D. M. Rothman and B. Imperiali, *Org. Biomol. Chem.*, 2004, **2**, 1965.





Outline of the peptide synthesis strategies

The sequences of the different peptides are:

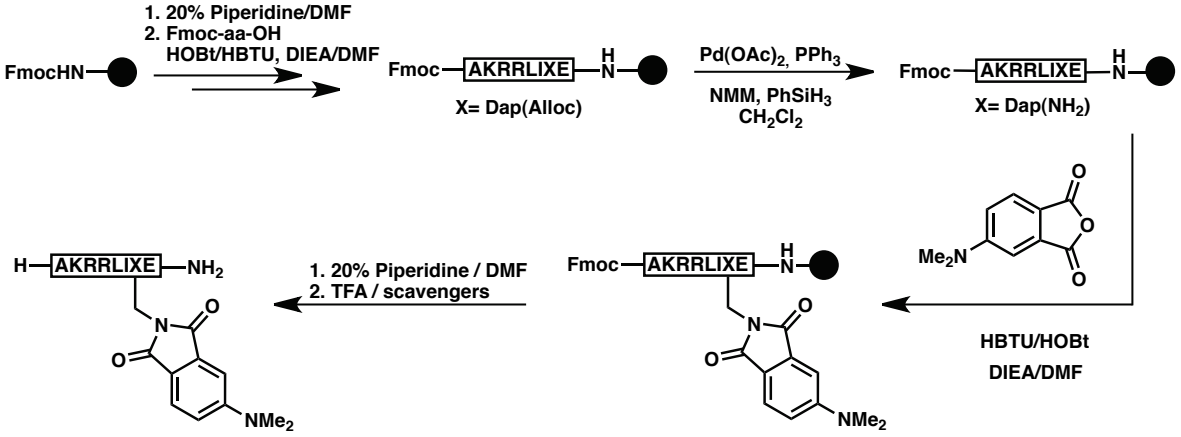
1a/1b – H_2N-AKRRLϕGE
2a/2b – H_2N-AKRRLϕFE
3a/3b – H_2N-AKRRGDϕE
4a/4b – H_2N-AKRRLGϕE
5a – H_2N-AKRRLIϕE
6a – H_2N-ϕAKRRLIFE
7a – ϕ-AKRRLIFE

Scheme S2. Sequences of fluorescent peptides.

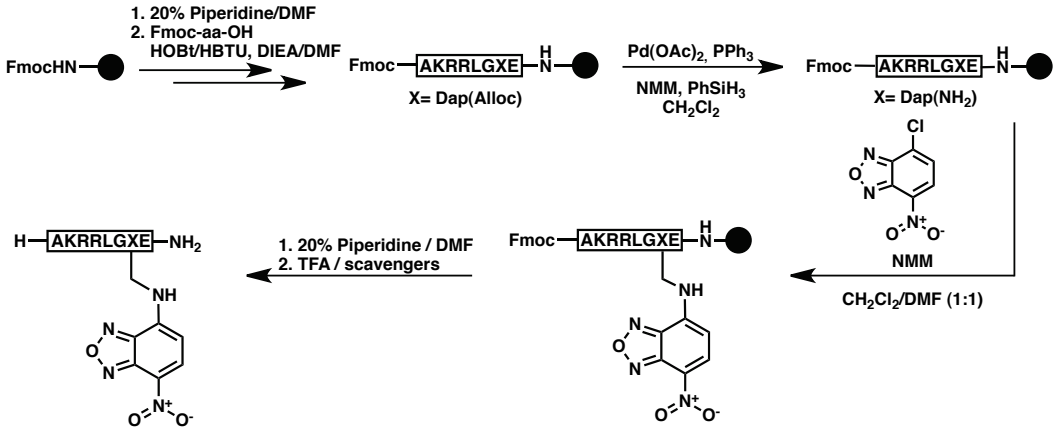
I1 – H_2N-HAKRRLFG
I2 – H_2N-SAKRRLFG
I3 – H_2N-HSKRRLFG

Scheme S3. Sequences of the inhibitors.

The strategy for the synthesis of side-chain fluorophore-conjugated peptides involved a modified protecting group scheme of the standard Fmoc solid phase peptide synthesis tactic. The 2,3-diaminopropionic acid (Dap) residue is introduced as an alloc-protected derivative, which allowed its selective side chain deprotection (Pd catalysis) and derivatization with the corresponding fluorophore.



Scheme S4. Synthesis of peptide **5a** with the *N*,*N*-dimethylaminopthalimide unit orthogonally attached to the Dap side chain.



Scheme S5. Synthesis of peptide **4b** with the NBD unit orthogonally attached to the Dap side chain.

Deprotection of the orthogonally protected Dap(Alloc) side chain: Once the peptides were fully assembled in the solid phase, the side chain of the Dap(Alloc) residue is selectively deprotected for specific attachment of the fluorophore, following in all cases the same procedure: 0.05 mmol of peptide attached to the solid support were treated at room temperature for 12 h with a mixture of $Pd(OAc)_2$ (0.3 eq), PPh_3 (1.5 eq), *N*-methylmorpholine (10 eq) and $PhSiH_3$ (10eq) in CH_2Cl_2 (2.5 mL). The resin was then filtered and washed with THF (1 × 5 mL × 2 min), DMF (2 × 5 mL × 2 min), diethyldithiocarbamate (DEDTC) (25 mg in 5 mL of DMF, 2 × 5 min), DMF (2 × 5 mL × 2 min) and CH_2Cl_2 (2 × 5 mL × 2 min).

Cleavage and deprotection of semipermanent protecting groups: The resin-bound peptide dried overnight (0.025 mmol) was placed in a 50 mL falcon tube, to which 3 mL of the cleavage cocktail (150 µL of CH_2Cl_2, 75 µL of water, 75 µL of triisopropylsilane (TIS) and TFA to 3 mL) were added, and the resulting mixture was shaken for 3 h. The resin was filtered, and the TFA filtrate was concentrated under argon current to a volume of approximately 2 mL. The residue was added to ice-cold diethyl ether (40 mL). After 10 min, the precipitate was centrifuged and washed again with 20 mL of ice-cold ether. The solid residue was dried under argon and redissolved in acetonitrile/water 1:1 (2 mL) and purified by preparative reverse-phase HPLC. The collected fractions were lyophilized and stored at −20 °C.

Peptides 1a-7a. 4-*N*,*N*-dimethylaminophthalic anhydride coupling to Dap side chain: 4-*N*,*N*-dimethylaminophthalic anhydride (40 mg, 0.2 mmol) was dissolved in DIEA/DMF 0.195M (2mL), and added over the Alloc-deprotected peptide attached to the resin (0.05 mmol) and was shaken for 30 min. Over the resin was then added HBTU/HOBt/DMF 0.2M (250 µL, 0.05mmol) and was shaken overnight. The resin was washed with DMF (3 × 3 mL × 3 min) and subjected to

the final Fmoc deprotection step using standard conditions (20% piperidine/DMF). HPLC purification of the crude gave a yellow solid, which was identified as the desired product by MS.

1a: H_2N–Ala–Lys–Arg–Arg–Leu–Dap(4-DMAP)–Gly–Glu–$CONH_2$, $C_{47}H_{78}N_{18}O_{12}$, ESI-MS [MH^+] calcd 1087.60, found 1087.4.

2a: H_2N–Ala–Lys–Arg–Arg–Leu–Dap(4-DMAP)–Phe–Glu–$CONH_2$, $C_{54}H_{84}N_{18}O_{12}$, ESI-MS [MH^+] calcd 1177.65, found 1177.4.

3a: H_2N–Ala–Lys–Arg–Arg–Gly–Asp–Dap(4-DMAP)–Glu–$CONH_2$, $C_{45}H_{72}N_{18}O_{14}$, ESI-MS [MH^+] calcd 1089.54, found 1089.8.

4a: H_2N–Ala–Lys–Arg–Arg–Leu–Gly–Dap(4-DMAP)–Glu–$CONH_2$, $C_{47}H_{78}N_{18}O_{12}$, ESI-MS [MH^+] calcd 1087.60, found 1087.4.

5a: H_2N–Ala–Lys–Arg–Arg–Leu–Ile–Dap(4-DMAP)–Glu–$CONH_2$, $C_{51}H_{86}N_{18}O_{12}$, ESI-MS [MH^+] calcd 1143.67, found 1143.2.

6a: H_2N–Dap(4-DMAP)–Ala–Lys–Arg–Arg–Leu–Ile–Phe–Glu–$CONH_2$, $C_{60}H_{95}N_{19}O_{13}$, ESI-MS [MH^+] calcd 1290.73, found 1290.6.

7a: (4-DMAP)N–Gly–Ala–Lys–Arg–Arg–Leu–Ile–Phe–Glu–$CONH_2$, $C_{59}H_{93}N_{19}O_{13}$, ESI-MS [MH^+] calcd 1261.71, found 1261.4.

Peptides 1b-4b. 4-chloro-7-nitrobenzofurazan coupling to Dap side chain: 4-chloro-7-nitrobenzofurazan (60 mg, 0.3 mmol) was dissolved in CH_2Cl_2/DMF (1:1) (3mL), and added over the Alloc-deprotected peptide attached to the resin (0.03 mmol). Then NMM (33 µL, 0.3mmol) was added and the mixture was shaked overnight. The resin was washed with DMF (3 × 3 mL × 3 min) and subjected to the final Fmoc deprotection step using standard conditions (20% piperidine/DMF). HPLC purification of the crude yielded a white solid identified as the desired product.

1b: H_2N–Ala–Lys–Arg–Arg–Leu–Dap(NBD)–Gly–Glu–$CONH_2$, $C_{43}H_{72}N_{20}O_{13}$, ESI-MS [MH^+] calcd 1077.56, found 1077.3.

2b: H_2N–Ala–Lys–Arg–Arg–Leu–Dap(NBD)–Phe–Glu–$CONH_2$, $C_{50}H_{78}N_{20}O_{13}$, ESI-MS [MH^+] calcd 1167.61, found 1167.5.

3b: H_2N–Ala–Lys–Arg–Arg–Gly–Asp–Dap(NBD)–Glu–$CONH_2$, $C_{41}H_{66}N_{20}O_{15}$, ESI-MS [MH^+] calcd 1079.50, found 1079.4.

4b: H_2N–Ala–Lys–Arg–Arg–Leu–Gly–Dap(NBD)–Glu–$CONH_2$, $C_{43}H_{72}N_{20}O_{13}$, ESI-MS [MH^+] calcd 1077.56, found 1077.4.

Peptides I1-I3: The final Fmoc deprotection step was carried out using standard conditions and the HPLC purification of the crude gave white solids, identified by MS as the desired products.

I1: H_2N–His–Ala–Lys–Arg–Arg–Leu–Phe–Gly–$CONH_2$, $C_{44}H_{74}N_{18}O_8$, ESI-MS $[MH^+]$ calcd 983.59, found 984.0.

I2: H_2N–Ser–Ala–Lys–Arg–Arg–Leu–Phe–Gly–$CONH_2$, $C_{41}H_{72}N_{16}O_9$, ESI-MS $[MH^+]$ calcd 933.57, found 933.7.

I3: H_2N–His–Ser–Lys–Arg–Arg–Leu–Phe–Gly–$CONH_2$, $C_{44}H_{74}N_{18}O_9$, ESI-MS $[MH^+]$ calcd 999.59, found 999.7.

Cyclin expression and purification:[2] Cyclin A was expressed and purified following reported procedures. Full-length human Cyclin A open reading frame was cloned into pET28a and was expressed in *Escherichia coli* BL21(DE3). Cells were grown at 37 °C to mid-log phase (A600 = 0.6). Expression was induced by the addition of IPTG (isopropyl 1-thio-β-D-galactopyranoside) 0.1 mM, and the culture was incubated for 4 h at room temperature. Bacteria were harvested by centrifugation at 4,500 rpm for 15 min, and the cell pellet was frozen at –80 ºC overnight. The pellet was resuspended in NENT buffer (20 mM Tris pH 8, 0.5M NaCl, 10% glicerol, 10mM b-Mercaptoetanol) with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 g/ml aprotinin, and 10 g/mL leupeptin). Cells were lysed by sonication avoiding overheating. The insoluble fraction was pelleted by centrifugation and the supernatant was purified with a His Trap HP column according to the manufacturer's instructions. The protein sample was concentrated to 3-4 mL by centrifugation using an *Amicon 15* (cut-off 5000) before being subjected to ionic exchange chromatography with a *Hi Trap Q HP* 1 mL column. The protein sample was desalted with a *GE Healthcare* PD-10 column using PBS pH 7.3 and final concentration was determined by UV spectroscopy (ε_{280} = 41,830 M^{-1} cm^{-1}).

2 X. Wang, M. Fu, J. Ren and X. Qu, *Protein expression and purification,* 2007, **56**, 27.

Fluorescence titration of peptide 4a with Cyclin A. To 150 µL of a 1.5 µM solution of peptide **4a** in HEPES buffer (10 mM HEPES pH 7.6, 100 mM NaCl), 1 µL aliquots of a 97 µM stock solution of Cyclin A were successively added at 20 °C, and the fluorescence spectra were recorded after each addition.

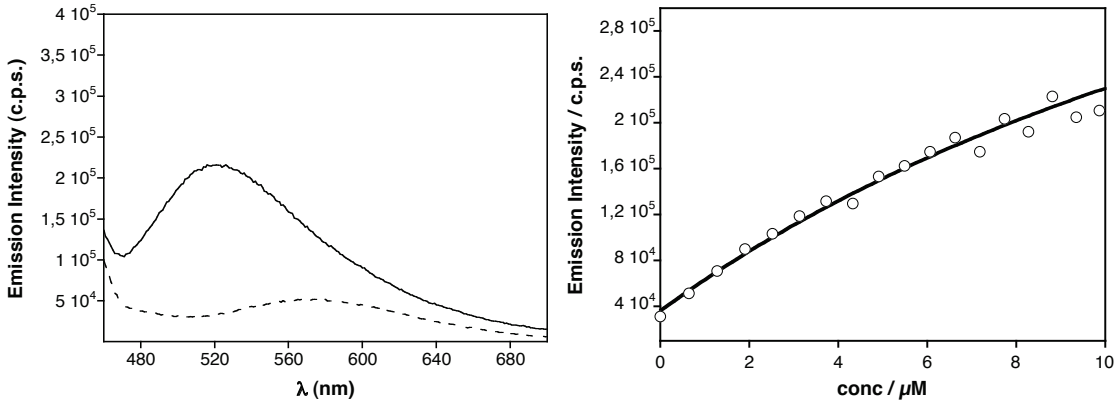


Figure S1 Left: Dashed line; 1.5 μM of peptide **4a** in 10 mM HEPES pH 7.6, 100 mM NaCl. Solid line; the same sample in the presence of Cyclin A ≈ 9.9 μM. **Figure 1 Right**: plot of the fluorescence emission intensity at 512 nm with the best-fitting binding curve.

Fluorescence spectra of peptide 4b with Cyclin A. To 130 µL of a 1.5 µM solution of peptide **4b** in HEPES buffer (10 mM HEPES pH 7.6, 100 mM NaCl), 20 µL aliquot of a 19 µM stock solution of Cyclin A were added at 20 °C and the fluorescence spectra were recorded after each addition.

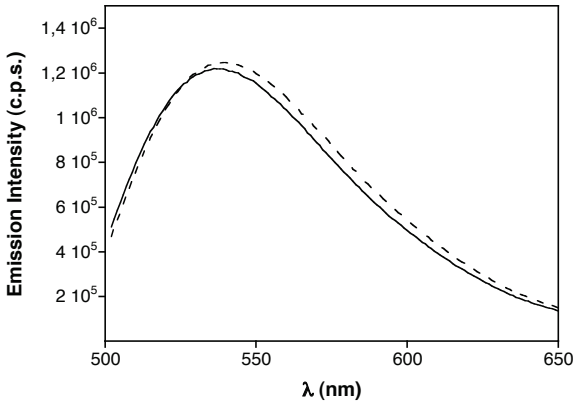


Figure S2: Dashed line; 1.5 μM of peptide **4b** in 10 mM HEPES pH 7.6, 100 mM NaCl. Solid line; the same sample in the presence of Cyclin A ≈ 2.5 μM.

Fluorescence titration of peptide 5a with Cyclin A. To 150 µL of a 1.5 µM solution of peptide **5a** in HEPES buffer (10 mM HEPES pH 7.6, 100 mM NaCl), 1 µL aliquots of a 97 µM stock solution of Cyclin A were successively added at 20 °C and the fluorescence spectra were recorded after each addition.

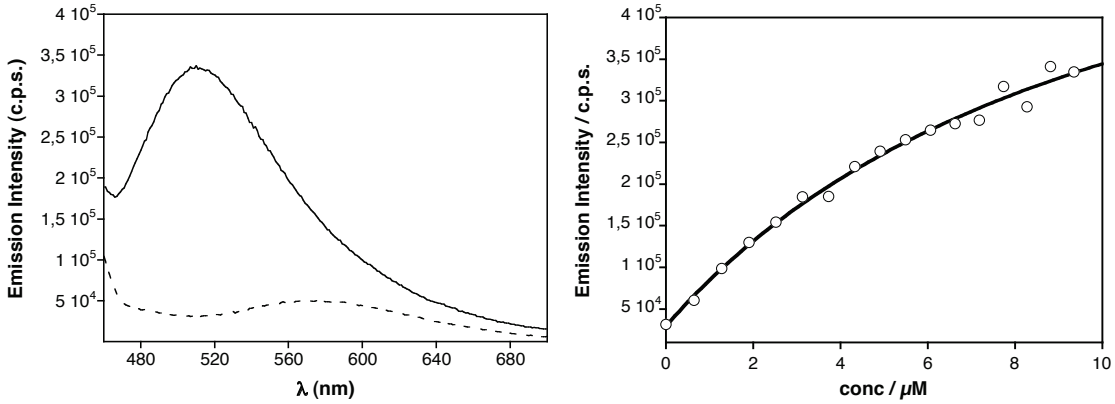


Figure S3 Left: Dashed line; 1.5 μM of peptide **5a** in 10 mM HEPES pH 7.6, 100 mM NaCl. Solid line; the same sample in the presence of Cyclin A ≈ 9.3 μM. **Figure 2 Right**: plot of the fluorescence emission intensity at 512 nm with the best-fitting binding curve.

Control experiments with other proteins. Variable volumes of stock protein solutions (IgG, Histone H1, BSA) were added to a final 10 µM concentration over 150 µL of a 1.5 µM solution of peptide **5a** in HEPES buffer (10 mM HEPES pH 7.6, 100 mM NaCl). The fluorescence spectra were recorded using the standard settings.

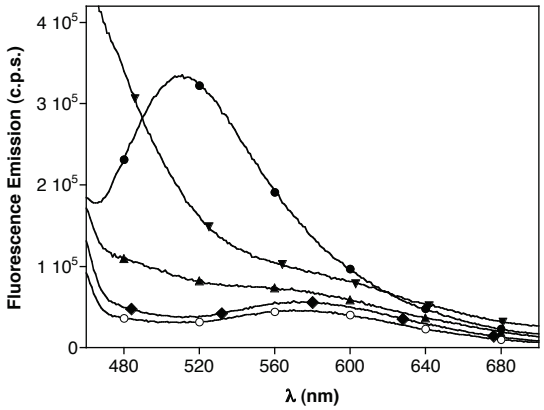


Figure S4. Left: Fluorescence emission of probe **5a** (1.5 μM) in 10 mM HEPES pH 7.6, 100 mM NaCl (○), and the same peptide in the presence of 10 μM Cyclin A (●); BSA (▲); IgG (▼); Histone H1 (♦).

Cyclin A groove polarity. The polarity of the Cyclin Binding Groove (CBG) was estimated from the Stokes shift of the probe **5a** under saturating concentrations of Cyclin A. The dependence of the shift with the polarity of the local environment is determined by measurements of fluorescence emission in solvents with various polarities.[3]

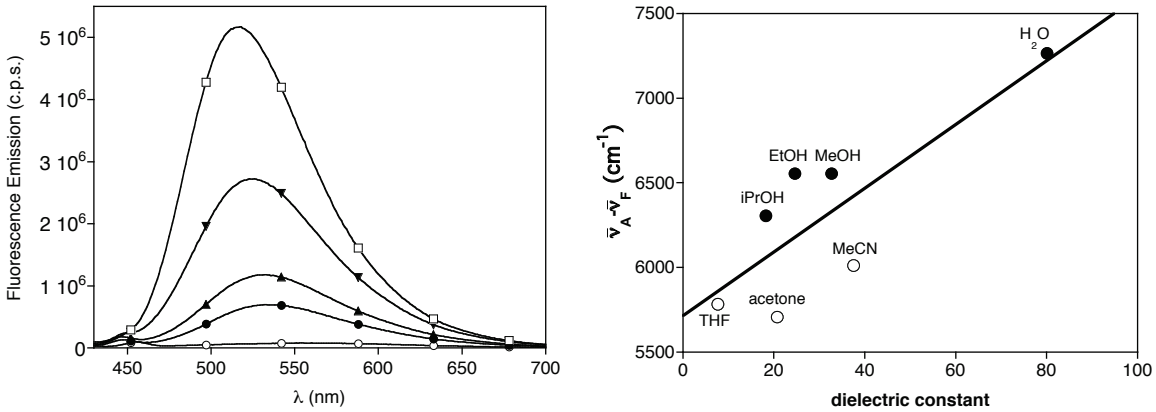


Figure S5. Left: Fluorescence emission of probe **5a** (1.5 μM) in different solvents: water (○); methanol (●); ethanol (▲); isopropanol (▼); acetonitrile (□). **Right**: Plot of the Stokes shift of **5a** (expressed as $1/\lambda_{em}-1/\lambda_{exc}$) vs. the dielectric constant in different solvents.

The Stokes shift, expressed as the difference between the absorption and excitation peaks expressed in wavenumbers (cm^{-1}), is plotted against the dielectric constant of each solvent, and a linear fit provides the relationship between both parameters. The resulting calibration line ($1/\lambda_{em} - 1/\lambda_{exc} = 5716 + 18.8 \times \varepsilon$) is used to obtain the polarity of the environment in the vicinity of the probe from the Stokes shift of the peptide bound to Cyclin A (~ 3.8).

[3] (*a*) S.-Y. Dong, H.-M. Ma, X.-J. Duan, X.-Q. Chen, J. Li, *J. Proteome Res.* 2005, **4**, 161; (*b*) T. Kimura, K. Kawai, T. Majima, *Org. Lett.* 2005, **7**, 5829; (*c*) Md. E. Haque, S. Ray, A. Chakrabarti, *J. Fluorescence* 2000, **10**, 1.

Competition titrations of peptide inhibitors with Cyclin A and peptide 5a. To 150 µL of a 1.5 µM solution of Cyclin A and peptide **5a** in HEPES buffer (10 mM HEPES pH 7.6, 100 mM NaCl), 1 µL aliquots of a 30 µM stock solution of the known peptide inhibitor **I1** were successively added at 20 °C, and the fluorescence spectra were recorded after each addition. This same procedure was followed for the competitions with **I2** and **I3**. See **data fitting** section below for curve fitting model and parameters.

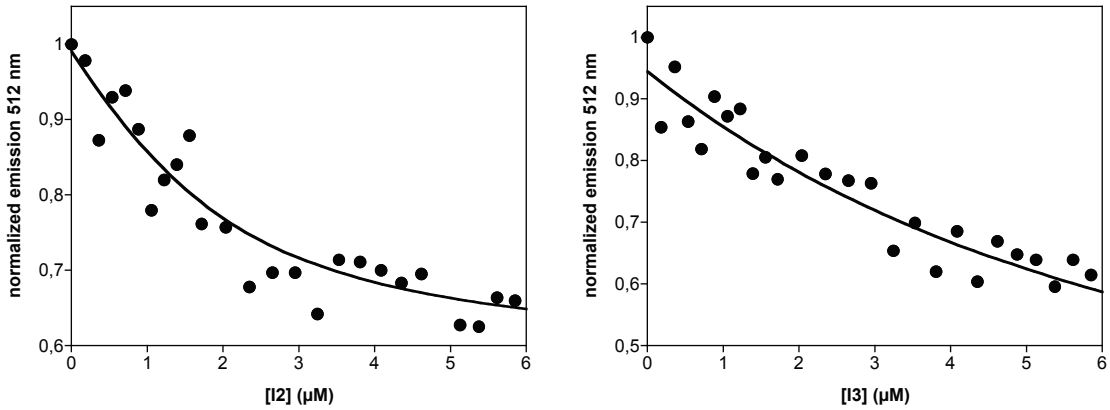


Figure S6. Competition titrations of 1.5 μM of peptide **5a** in 10 mM HEPES pH 7.6, 100 mM NaCl, and 1.5 μM Cyclin A with increasing amounts of peptide inhibitors. Solid line corresponds to the best fitting according to the model presented in the **data fitting** section **Left:** peptide inhibitor **I2; Right**: peptide inhibitor **I3**.

Peptide internalization studies. For live-cell imaging, HeLa cells (1 x 10^5 cells/ well) were seeded in 6-well culture plates and left to adhere for 24 h. Cells were then incubated for 2 hours in DMEM medium containing the peptide **5a** (20 µM). Before visualization, medium containing the peptide was removed from the cells by aspiration, and the cells were washed three times with PBS (37 ºC). Images were captured with a *Leica* confocal microscope equipped with a l-blue 40× objective and handled with a TCS SP2 system, equipped with an Acoustic Optical Beam Splitter (AOBS). Excitation was with an argon laser (548 nm, 476 nm, 488 nm, 496 nm and 514 nm) and blue diode (405 nm). Images were captured at an 8-bit gray scale and processed with LCS software (version 2.5.1347a, *Leica* Germany) containing multicolor, macro and 3D components.

MTT cell viability assay. HeLa cells were seeded in sterile 96-well microtiter plates at a seeding density of 1.5 × 10^5 cells/mL and allow settling for 24 h. The indicated

concentrations of peptide **5a** were then added to the media. After 19 hour of incubation MTT (20 µL of a 5 mg/mL solution) was added to each well, and the cells were further incubated for 5 h (a total of 24 h incubation with the inhibitors was therefore studied). After removal of the medium, the precipitated formazan crystals were dissolved in optical grade DMSO (100 µL), and the plates were read at 570 nm using a *Wallac* 1420 Workstation

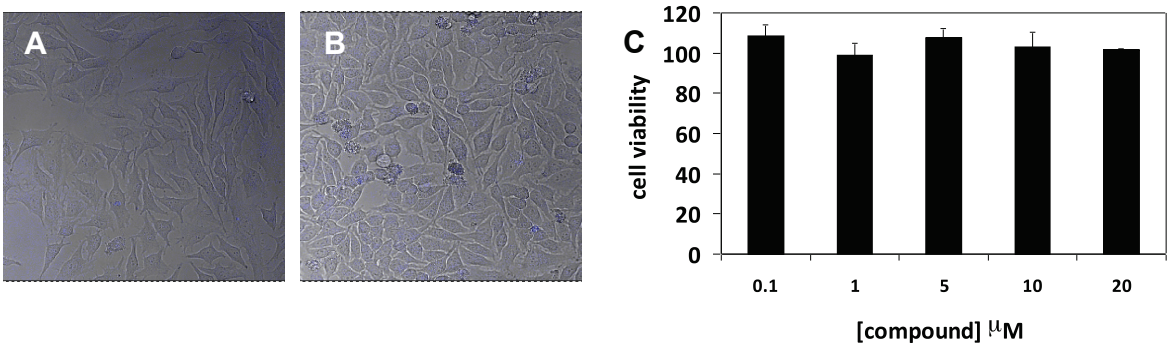


Figure S7. Cellular internalization of peptide **5a**. Confocal images of HeLa cells incubated during 2 hours either in the absence (A) or in the presence (B) of peptide **5a** at 20µM. (C) Cell viability assays of HeLa cells incubated with increasing concentrations of peptide **5a** for 24 hours.

Molecular modeling methods

The crystal structure of cyclin / CDK in complex with the peptidic inhibitor RRLIF-NH_2,[4] (PDB code 1OKV) was used as starting point to build the complex of cyclin with peptide **5a**. The transformation of the peptide RRLIF-NH_2 into the sequence H_2N-AKRRLIΦE was done with the *Schrödinger* suite of programs, by means of the *Maestro* graphical interface and its builder module. After deletion of the CDK protein from the complex, hydrogens were added and a partially restrained Energy minimization followed with the module *Macromodel* of the *Schrödinger* suite.[5] This consisted in 1000 steps of Polack-Ribiere-conjugated gradients algorithm, considering aqueous continuum solvent (GB model). The OPLS forcefield,[6] as implemented in *Macromodel* was employed, the Ca atoms of the protein were frozen, and the three hydrogen bonds achieved between

4 G. Kontopidis, M. J. I. Andrews, C. McInnes, A. Cowan, H. Powers, L. Innes, A. Plater, G. Griffiths, D. Paterson, D. I. Zheleva, D. P. Lane, S. Green, M. D. Walkinshaw and P. M. Fischer, *Structure*, 2003, **11**, 1537.

5 *MacroModel version 9.6*; Schrödinger, LLC: NY, 2005.

6 G. A. Kaminski, R. A. Friesner, J. Tirado-Rives and W. L. Jorgensen, *J. Phys. Chem. B,* 2001, **105**, 6474.

the peptide and residues Gln^{254}, Glu^{220} in the CBG were restrained by 100 Kcal/mol·Å^2 force constant.

The resulting Cyclin/**5a** complex was used as input for extensive molecular dynamics (MD) simulations with the program NAMD[7]. Preparation and analysis were performed with the VMD software,[8] and the OPLS forcefield[6] was used for the simulations. Missing parameters for the 4-DMAP moiety in the ligand were obtained from the previous step with *Macromodel*, and implemented into the NAMD version of the forcefield by an *ad hoc* script. The system was immersed in a box of TIP3 waters of approximately 70 × 70 × 82 Å^3 in such a way that a minimum distance of 12 Å was maintained between the most distal atom of the solute and the limits of the solvent box. Periodic boundary conditions were used in the NPT ensemble, and the Particle Mesh Ewald method[9] was adopted for the treatment of long-range electrostatic forces beyond a 12 Å cutoff. An initial 1000 step energy minimization phase was followed by an initial smooth equilibration period of 50 ps, where the temperature was raised from 1 to 310 K, while temperature bath was relaxed (Langevin damping from 5/ps till a final value of 0.1/ps) and a tiny time step of 1 fs was employed. Initial positional restraints were imposed to the Cα trace through a force constant of 10 Kcal/mol·Å^2, which were gradually released through this smooth equilibration phase. A second equilibration phase followed for 1 ns, where all hydrogen bonds between protein and the main chain of the peptide (in an initial number of 10) were restrained with a stepwise decreasing force constant (from 50 to 5 Kcal/mol·Å^2). A 2fs time step was already used in this phase, allowing the treatment of all bonds of hydrogen atoms with the SHAKE algorithm.[10] Unrestrained MD followed for 5 ns, which constitutes the collection period used for analysis. Such analysis was performed with VMD standard tools: hydrogen bonds were computed with the *hbonds* plugin, with maximum distance of 3.2 Å between heteroatoms and a minimum angle of 120 deg. The RMSD trajectory tool and solvent-accessible surface area calculations were performed with the corresponding plugins using default settings.

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Data fitting. The titration data for peptides **4a** and **5a** with Cyclin A were fit to a simple 1:1 model for a fluorescently-labeled ligand binding to a unlabeled receptor. [11]

$$(1) \qquad F_T = \frac{1}{2}\left(2F_0 + F_{RL} \times \left[K_D + L_T + R_T - \sqrt{\left(K_D + L_T + R_T\right)^2 - 4L_T R_T}\right]\right)$$

Where $\boldsymbol{R_T}$, total receptor concentration; $\boldsymbol{L_T}$, total concentration of the labeled ligand; $\boldsymbol{K_D}$, dissociation constant of the interaction between the receptor and the ligand; $\boldsymbol{F_T}$, total observed fluorescence, $\boldsymbol{F_0}$, adjustable parameter accounting for the background fluorescence; $\boldsymbol{F_{RL}}$ adjustable parameter for the labeled ligand-receptor complex molar fluorescence. Data fitting was made with *Mathematica*® 8.0.1.0 program for MacOS X (Wolfram Research).

The experimental data of the competition assays of the complex **5a** - Cyclin A with the inhibitors were fit to model for an unlabeled ligand, $\boldsymbol{L}$, and a fluorescently labeled ligand, $\boldsymbol{L_S}$, that compete for binding to a unlabeled receptor, $\boldsymbol{R}$. [11]

$$(2) \qquad F_T = F_0 + \left(F_{\max} - F_0\right)\frac{2\sqrt{\left(a^2 - 3b\right)}\cos\left(\theta/3\right) - a}{3K_{D1} + 2\sqrt{\left(a^2 - 3b\right)}\cos\left(\theta/3\right) - a}$$

Where:

$$\theta = \arccos\left[\frac{-2a^3 + 9ab - 27c}{2\sqrt{\left(a^2 - 3b\right)^3}}\right]$$

$$a = K_{D1} + K_{D2} + L_{ST} + L_T - R_T$$

$$b = \left(L_T - R_T\right)K_{D1} + \left(L_{ST} - R_T\right)K_{D2} + K_{D1}K_{D2}$$

$$c = -K_{D1}K_{D2}R_T$$

Where $\boldsymbol{R_T}$, total receptor concentration; $\boldsymbol{L_{ST}}$, total concentration of the labeled ligand; $\boldsymbol{L_T}$, total concentration of the unlabeled ligand; $\boldsymbol{K_{D1}}$, dissociation constant of the interaction between the receptor and the labeled ligand; $\boldsymbol{K_{D2}}$, dissociation constant of the interaction between the receptor and the unlabeled ligand; $\boldsymbol{F_T}$, total observed fluorescence; $\boldsymbol{F_0}$, adjustable parameter accounting for the background fluorescence; $\boldsymbol{F_{max}}$ adjustable parameter for the initial ligand-receptor complex.

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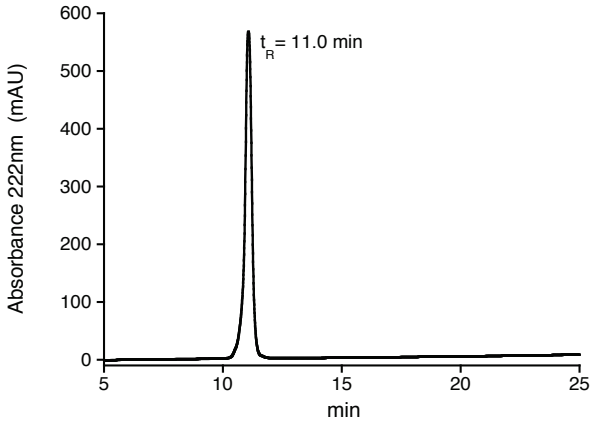
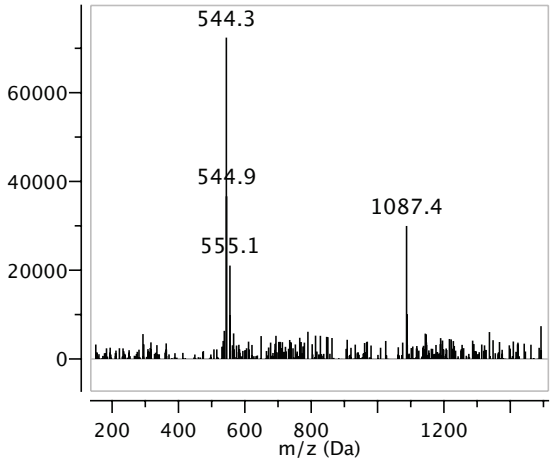
[11] (*a*) M. H. A. Roehrl, J. Y. Wang and G. Wagner, *Biochemistry,* 2004, **43**, 16056; (*b*) P. Thordarson, *Chem. Soc. Rev.*, 2011, **40**, 1305; (*c*) M. R. Eftink, *Methods Enzymol.*, 1997, **278**, 221; (*d*) G. Shi, Y. Gong, A. Savchenko, J. G. Zeikus, B. Xiao, X. Ji and H. Yan, *Biochim. Biophys. Acta*, 2000, **1478**, 289.

HPLC and MS-ESI spectra.

Peptide 1a.





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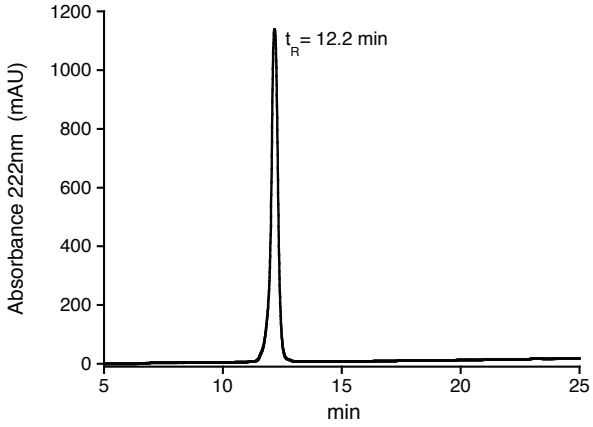

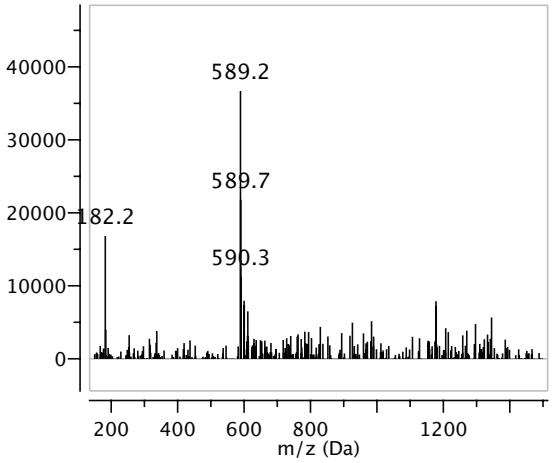

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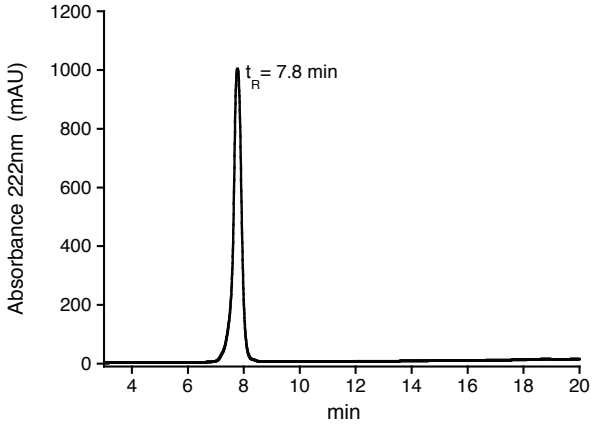

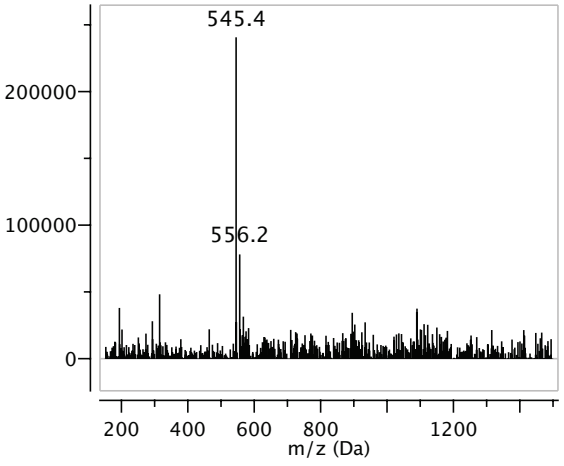

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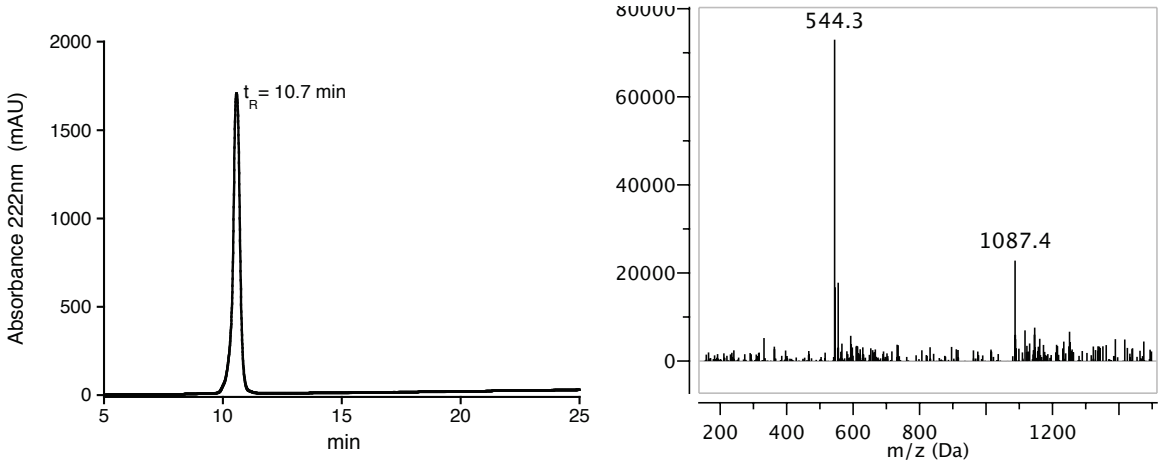

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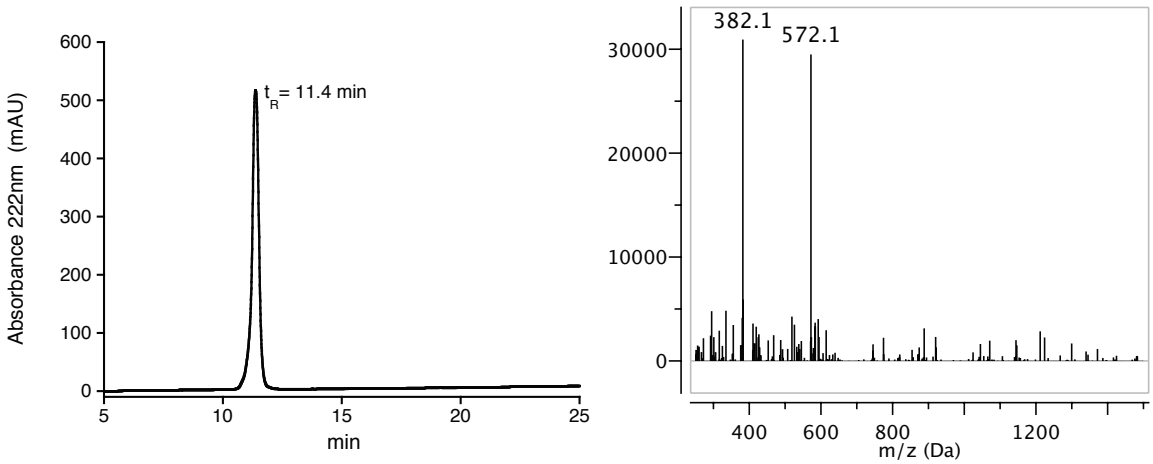

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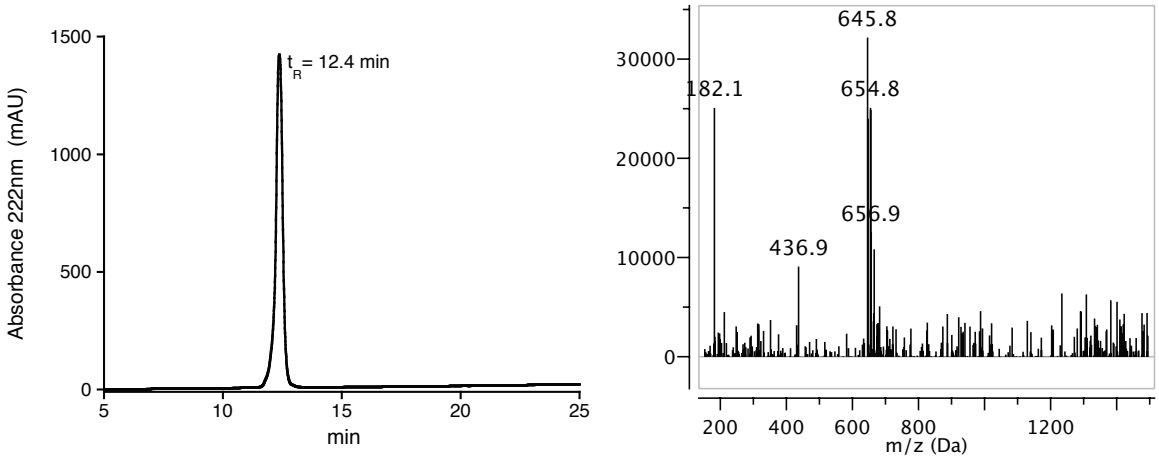

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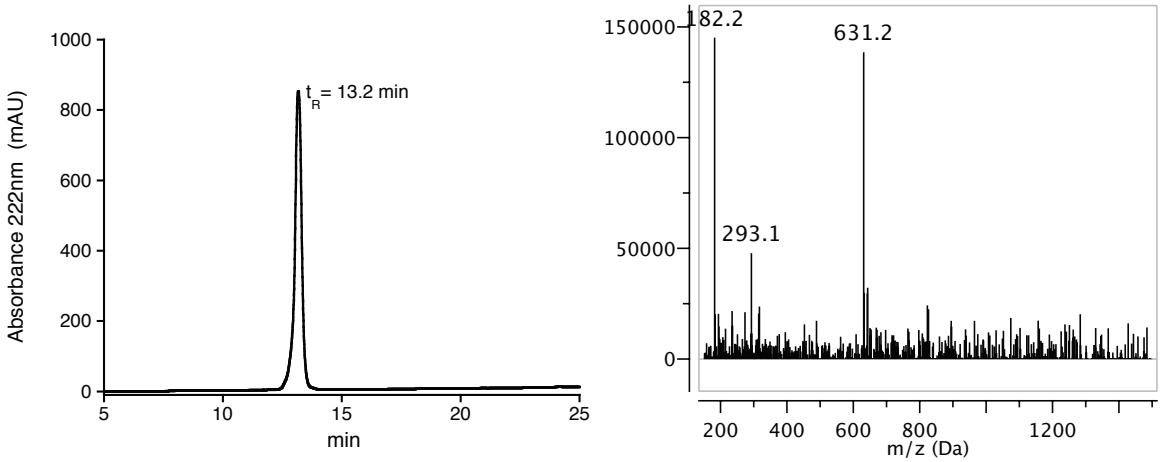

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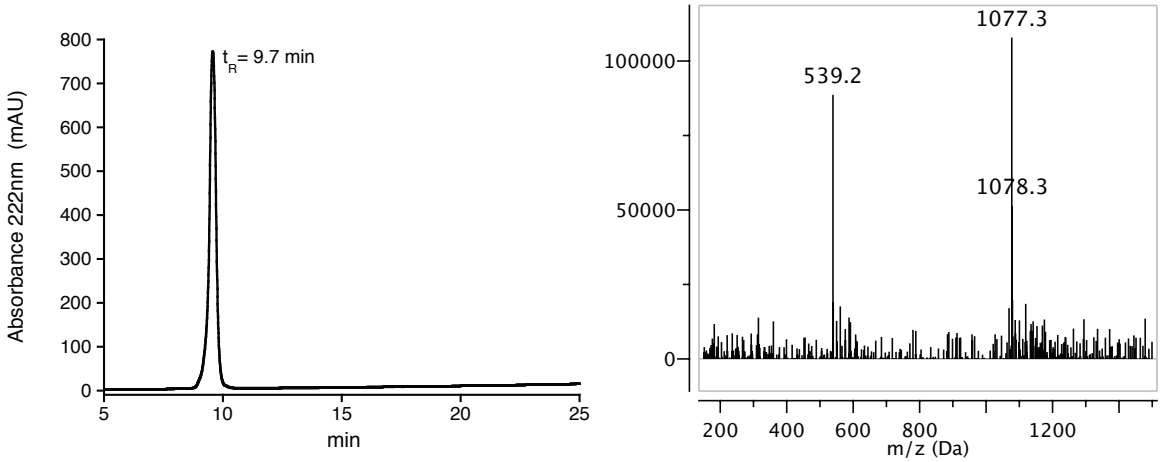

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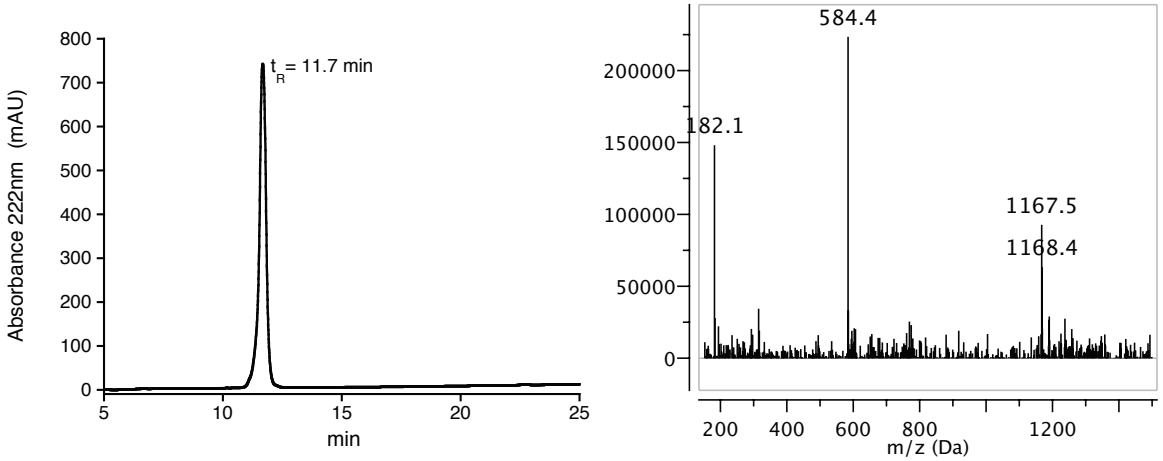

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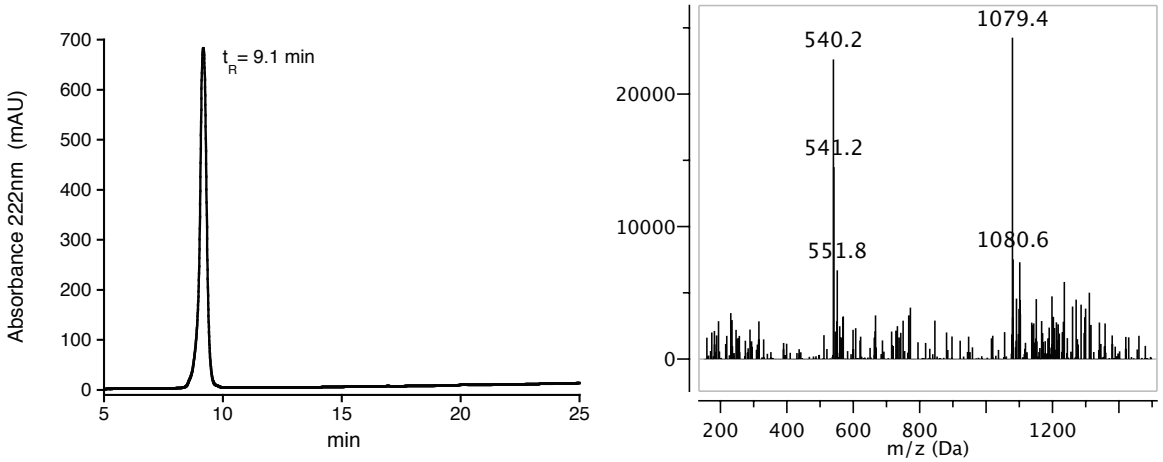

Peptide 4b.