Supporting information for:

Hairpin Peptide Beacon: Dual-labeled PNA-Peptide-Hybrids for protein detection

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1) Materials and general procedures

Materials: PNA monomers were purchased from Applied Biosystems/USA. PyBOP, HCTU, Rink-amide resin, the phosphotyrosine building blocks Fmoc-Tyr(PO(NMe₂)₂-OH and Fmoc-Lys(DabcyI)-OH were purchased from Novabiochem/Switzerland. Fmoc-Statine was purchased from neomps/France. Standard Fmoc/tBu protected amino acids were purchased from Senn chemicals/USA. NIR-664-succinimidylester and dry DMF (H₂O < 0.01%) were purchased from Fluka/Germany. Pyrenebutanoic acid and human renin were purchased from Sigma Aldrich/Germany. NMP, Piperidine and TFA were purchased in peptide synthesis grade and used without further purification. Commercial reagents were used without further purifications. Water was purified with a Milli-Q Ultra Pure Water Purification System.

Overexpression and Purification of GST-Src-SH2: The Src-SH2 domain (ggSrc PubMed: aaa70194,142-246, N234T corresponding to human Src 145-249, A168P, N224S, T246N) was cloned into expression vector pGEX-4T1 to produce it as glutathione-S-transferase (GST) fusion protein in *Escherichia coli*. We purified GST-Src-SH2 by glutathione sepharose affinity chromatography, concentration with centrifugal concentrators (Vivaspin 10 kDa MWCO, Viva Science) and size exclusion chromatography (Superdex 75, Pharmacia, Uppsala, Sweden) to obtain a final purity of at least 98 %. The purified protein solution was aliquoted and stored at -80°C after freezing in liquid nitrogen. Thawed aliquots were stored at 0°C and used for a maximum of three days.

Solid-phase synthesis: Manual solid phase synthesis was performed by using 2 mL polyethylene syringe reactors that are equipped with a fritted disc. Automated linear solid-phase Fmoc-synthesis was performed by using an Intavis ResPep parallel synthesizer equipped with micro scale columns for PNA synthesis.

Preloading of resins: The resins used in solid phase synthesis were loaded with the protected amino acids (ca. 0.13 mmol/g) according to standard protocols (see: *NovaBiochem Catalog 2004/2005*).

HPLC: Analytical HPLC-MS was by using an Agilent 1100 HPLC-MS system equipped with a UV/Vis-detector and a VL-quadrupole mass spectrometer using a thermostated (55 °C) analytical CC 125/4 Nucleodur-C18 gravity, 3μ column (Macherey-Nagel) and a detection wavelength λ=260 nm. Eluents A (H₂O:MeCN:HCOOH = 98.9:1:0.1 (v:v:v)) and B (MeCN:H₂O:HCOOH = 98.9:1:0.1 (v:v:v)) were used in a linear gradient at a flow rate of 1 mL/min.

Gradient A: 03-80 % B in 30 min.

Gradient B: 15-80 % B in 30 min.

Preparative HPLC was performed on an Agilent 1100 HPLC system equipped with a multi wavelength detector using a semipreparative 250/10 C18-Polaris, 5 μ column (Varian) and a detection wavelength λ =280 nm. Eluents A (H₂O:MeCN:TFA = 98.9:1:0.1 (v:v:v)) and B (MeCN:H₂O:TFA = 98.9:1:0.1 (v:v:v)) were used in a linear gradient from 15 % B to 80 % B in 30 minutes at a flow rate of 6 mL/min.

Automated solid-phase synthesis according to the Fmoc-strategy: Automated solid phase synthesis of HPBs was performed in a 2 μ Mol scale using a Fmoc-Glycine preloaded tentagel Rink resin (NovaSyn TGR, Novabiochem) with a loading of 0.15 mmol/g, Fmoc/Bhoc-protected PNA-monomers, Fmoc-Lys(DabcyI)-OH, Fmoc-Statine-OH **7** (figure S1) and Fmoc/tBu-protected amino acids (Senn Chemicals). Phosphotyrosine was coupled as Fmoc-Tyr(PO(NMe₂)₂)-OH. For coupling of PNA-monomers the desired monomer was preactivated for 1 minute using 4 eq. of monomer, 3.6 eq. HCTU and 8 eq. *N*-methylmorpholine. Amino acids were preactivated for 30 seconds using 6 eq. amino acid, 5.4 eq. HCTU and 12 eq. *N*-methylmorpholine. Double couplings were used after assembly of the C-terminal PNA-segment. After completion of the linear assembly the probe was transferred to a 2 mL polyethylene syringe reactor for attaching the desired fluorophores. The Mmt-protecting group of lysine was removed by treatment with a solution of CH₂Cl₂/TIS/TFA (93:5:2/v:v:v) for 2 min, followed by washing with 3xCH₂Cl₂, 2x pyridine, 3xDMF, 3xCH₂Cl₂ and 3xDMF. The NIR-fluorophore (when needed) was incorporated as NIR-664 succinimidylester **8** (figure S1) by double coupling of 10 eq. NIR-664 SE and using 20 eq. of *N*-methylmorpholine. The pyrene moities were incorporated as pyrenebutanoic acid **9** (figure S1) by double coupling of 20 eq. pyrenebutanoic acid, using 20 eq. PyBOP, 40 eq. of *N*-methylmorpholine and in situ activation. After completion of the linear synthesis the probe cleavage was induced upon treatment with 1 mL TFA/H₂O/TIS/*m*-cresole (85:5:5:5/v:v:v:v). The resin was washed twice with 200 μ L TFA. For the removal of the phosphotyrosine protecting group 10-Vol % H₂O was added to the solution. After shaking for 12-16 h the solution was concentrated under reduced pressure and the crude product precipitated with diethyl ether. After purification by preparative HPLC the product was characterized by analytical HPLC-MS.

Figure S1. Structures of used building blocks and fluorophores in solid phase synthesis.

Determination of yields: Yields of HPB synthesis have been calculated by using the extinction coefficients given below. The extinction coefficient of dabcyI and pyrenebutanoic acid was taken from literature (Invitrogen: Web edition of “The Handbook – A Guide to Fluorescent Probes and Labeling Technologies, Tenth edition”, (DabcyI: Data Table –1.8 Reagents of analysis of low molecular weight amines; pyrenebutanoic acid: Data Table –13.2 Fatty acid analogs and phospholipids)

Ac-Lys(pyrene)-ttgg-Glu-Pro-Gln-pTyr-Glu-Glu-Ile-Pro-ccaa-Lys(pyrene)-Gly-NH₂ (1)

Yield: (2 pyrene moities) 13.9 OD₃₄₀ (141 nMol, 7 %); $\epsilon_{340} = 43,000 \text{ M}^{-1} \cdot \text{cm}^{-1}$

HPLC/ESI-MS:	calculated:	4146.63
	found:	4146.33
	t _R :	4.51 min (Gradient B)

C₁₈₇H₂₃₀N₆₁O₄₉P (4146.63)

Ac-Lys(NIR)-ttgg- Pro-His-Pro-Phe-His-Statine-Val-Ile -ccaa-Lys(DabcyI)-Gly-NH₂ (2)

Yield: 37.1 OD₄₅₃ (394 nMol, 19 %); $\epsilon_{453} = 32,000 \text{ M}^{-1} \cdot \text{cm}^{-1}$

HPLC/ESI-MS:	calculated:	4385.14
	found:	4385.29
	t _R :	6.49 min (Gradient B)

C₂₀₄H₂₆₃N₆₉O₄₂S (4385.14)

Ac- Lys(pyrene)-gcta-Glu-Pro-Gln-pTyr-Glu-Glu-Ile-Pro-ccaa-Lys(pyrene)-Gly-NH₂ (3)

Yield: (2 pyrene moities) 1.4 OD₃₄₀ (1.5 nMol, 1 %); $\epsilon_{340} = 43,000 \text{ M}^{-1} \cdot \text{cm}^{-1}$

HPLC/ESI-MS:	calculated:	4116.17
	found:	4116.97
	t _R :	6.357 min (Gradient A)

C₁₈₆ H₂₂₉ N₆₂ O₄₇ P₁ (4116.17)

Ac-Lys(pyrene)-Glu-Pro-Gln-pTyr-Glu-Glu-Ile-Pro-Lys(pyrene)-Gly-NH₂ (4)

Yield: (2 pyrene moities) 18.2 OD₃₄₀ (207 nMol, 10 %); $\epsilon_{340}= 43,000 \text{ M}^{-1}\cdot\text{cm}^{-1}$

HPLC/ESI-MS:

calculated: 1979.12

found: 1979.43

t_R: 16.47 min (Gradient B)

C₁₀₁ H₁₂₄ N₁₅ O₂₅ P₁ (1979.12)

Ac- Lys(NIR)- Pro-His-Pro-Phe-His-Statine-Val-Ile- Lys(dabcyl)-Gly-NH₂ (5)

Yield: 20.0 OD₄₅₃ (213 nMol, 11 %); $\epsilon_{453}= 32,000 \text{ M}^{-1}\cdot\text{cm}^{-1}$

HPLC/ESI-MS:

calculated: 2217.41

found: 2217.52

t_R: 8.412 min (Gradient A)

C₁₁₈ H₁₅₇ N₂₃ O₁₈ S₁ (2217.41)

Ac- Pro-His-Pro-Phe-His-Statine-Val-Ile-NH₂ (6)

Yield: 4.1 mg (3.7 μ Mol, 74 %)

HPLC/ESI-MS:

calculated: 1101.30

found: 1101.18

t_R: 10.716 min (Gradient A)

C₅₄ H₈₀ N₁₄ O₁₁ (1101.30)

2) Src-SH2 as target

a) Fluorescence measurements

Fluorescence spectra and time courses for measurements with Src-SH2 were recorded using a Varian Cary Eclipse spectrometer. Measurements were carried out in fluorescence quartz cuvettes (4x10 mm) in a buffered solution (20 mM NaH₂PO₄, 100 mM NaCl, 2 mM DTT).¹ The buffer was degassed and the pH adjusted to 7.4 by using a degassed 2 M NaOH solution. Subsequent manipulations were carried out while avoiding unnecessary exposure to oxygen. To 1 mL of buffer was added **1** from a 0.1 mM stock solution in buffer to a final concentration of 1 μ M. To this solution Src-SH2 was added from a 1.6 mM stock solution to a final concentration of 16 μ M.

Measurements in scan mode: The spectrometer settings were selected as followed:

pyrenebutyl fluorescence: λ -ex = 340 nm (slit 5 nm), λ -em = 365-580 nm (slit 5 nm)

Measurements in kinetic mode: The spectrometer settings were selected as followed:

pyrenebutyl fluorescence: λ -ex = 340 nm (slit 5 nm), λ -em = 380 nm (slit 5 nm)

b) Thermal denaturation experiments

Denaturation experiments were performed on a Varian Cary Eclipse spectrometer in fluorescence mode (λ_{ex} : 340 nm, λ_{em} : 380 nm). Thermal denaturation was explored at different probe concentrations ranging from 0.625 μM to 5 μM in a freshly degassed 20 mM NaH_2PO_4 , 100 mM NaCl, 2mM DTT buffer at pH 7.4 and a heating rate of 0.5 $^\circ\text{C}/\text{min}$ (figure S2a). The given T_M -value is the mean of the maximum of the first derivative of at least three denaturation curves (figure S2b). $T_M = 60 \pm 2^\circ\text{C}$ was found to be independent of the probe concentration, which suggests intramolecular hybridization.

Figure S2. (a) Thermal denaturation curves of HPB (**1**) in four different concentrations. (b) Derivates of thermal denaturation curves.

c) Controll experiment

The pyrene units in HPB **1** are hydrophobic. It was thus considered necessary to investigate the fluorescence of **1** in response to addition of proteins that are known to bind nonpolar compounds. Bovine serum albumin (BSA) is such protein, that may be able to open the hairpin structure of **1**. Addition of BSA to a 0.5 μM solution of HPB **1** in buffer (20 mM NaH_2PO_4 , 100 mM NaCl, 2mM DTT at pH 7.4) resulted in minor increases of fluorescence emission at 380 nm. Importantly the responsiveness of HPB **1** in the presence of target protein Src-SH2 was not affected, since BSA increased the fluorescence of both, unbound and target-bound HPB **1** (figure S3).

Figure S3. Influence of BSA on fluorescence intensities of HPB **1** in absence and presence of Src-SH2. Conditions: 0.5 μ M HPB **1**, 8 μ M Src-SH2, 0.1 Vol% BSA, buffer: 20 mM NaH₂PO₄, 100 mM NaCl, pH 7.4, 25°C.

d) Determination of K_D - values

Determination of binding dissociation constants (K_D 's) were performed on a SPEX Fluoromax 3 fluorescence spectrometer (HORIBA Jobin Yvon) equipped with a peltier thermostated single cell holder (set to 25°C) and automated titrator. Slits were set to yield an intensity of $1.0 \cdot 10^{-4}$ counts at start of experiment.

Measurements were carried out in fluorescence quartz cuvettes (4x10 mm) in a buffered solution (20 mM NaH₂PO₄, 100 mM NaCl, 2 mM DTT).¹ The buffer was degassed and the pH adjusted to 7.4 by using a degassed 2 M NaOH solution. Subsequent manipulations were carried out while avoiding unnecessary exposure to oxygen. To 1 mL of buffer was added **1**, **3** and **4** respectively from 1 μ M stock solutions in buffer to a final concentration of 20 nM. The solution was allowed to equilibrate at 25°C for 10 minutes.

For determination of K_D -values a 720 nM Src-SH2 (stock: 1.7 mM) and a 720 nM Grb2-SH2 solution (stock: 2.2 mM) respectively in buffer (20 mM NaH₂PO₄, 100 mM NaCl at pH 7.4) containing 20 nM probe (to avoid dilution effects) were used.

To the 20 nM probe solution the Src-SH2 and the Grb2-SH2 solutions respectively were automatically titrated in 2 μ l steps to a final protein concentration of 15 μ M. The mean values of triple experiments were fitted using equation 8.10 from "Fluorescence Polarization Technical Resource Guide - Analysis of FP binding data, chapter 8".² The corresponding graphs of **1** (top) and **4** (bottom) versus Src-SH2 (left) and Grb2-SH2 (right) are shown in figure S4.

Figure S4. Binding curves of **1** (top) and **4** (bottom) versus Src-SH2 (left) and Grb2-SH2 (right). The data shown is an average of three independent experiments.

The K_D -values are summarized in table S1. It could be shown that PB (**4**) binds with lower affinity to Src-SH2 than the corresponding HPB (**1**). This suggests that the PNA arm-segments in **1** might serve as a linker that can separate the bulky hydrophobic pyrene residues from the binding site. It could also be determined that HPB **1** binds with a more than two times lower affinity to Grb2-SH2 than to Src-SH2 where **4** shows almost no difference between both proteins. This proves the higher selectivity of our HPBs compared to PBs.

S1. K_D -values of HPB **1** and PB **4** versus Src-SH2 and Grb2-SH2

	K_D [μ M] vs. Src-SH2	K_D [μ M] vs. Grb2-SH2
HPB (1)	1.5	3.5
PB (4)	4.5	4.4

Besides **1** and **4** we investigated the binding affinity to Src-SH2 of the PNA-Peptide Beacon **3** which was also pyrene-labeled. Both PNA segments were constructed in a way that hybridization could be excluded. This was confirmed by thermal denaturation experiments (data not shown). We therefore considered **3** as unstructured protein probe. Binding studies revealed a K_D -value of 110 nM (Figure S5). This demonstrates quite clearly that PNA *per se* does not have any negative site effects on binding to Src SH2.

Figure S5. Binding curve of **3** versus Src-SH2 (average of three independent experiments).

3) Human renin as target

a) Fluorescence measurements

Fluorescence spectra and time courses for measurements with human renin were recorded using a SPEX Fluoromax 3 fluorescence spectrometer (HORIBA Jobin Yvon) equipped with a peltier thermostated single cell holder (set to 37 °C). Slits were set to yield an intensity of $1.0 \cdot 10^{-4}$ counts at start of experiment. Measurements were carried out in a fluorescence quartz cuvette (volume 30 μ l) in a buffered solution (50 mM TRIS, 100 mM NaCl, 1mM EDTA and 0.1% BSA). The buffer was degassed and the pH adjusted to 7.4 by using a degassed 2 M HCl solution. Subsequent manipulations were carried out while avoiding unnecessary exposure to oxygen. To 40 μ L of buffer was added **2** and **5** respectively from a 1 μ M stock solution in buffer to a final concentration of 100 nM. To this solution human renin was added from a 0.35 mM stock solution to a final concentration of 120 nM for **2** and 600 nM for **5**. It could be shown that a fluorescence enhancement of **2** by a factor of 8.2 has been obtained (figure S6a). PB (**5**) only gave a fluorescence enhancement of a factor of 3 (figure S6b) although even higher renin concentrations have been used. The latter was necessary to get saturation of signalling.

The different fluorescence enhancements of **2** and **5** might be due to different structures. While the HPB (**2**) still forms a hairpin controlled by PNA hybridization (confirmed by thermal denaturation experiments, data not shown) the PB (**5**) exists in a random coil structure due to the less flexible peptide sequence. Consequently the NIR fluorescence is more quenched in **2** than in **5** which leads to higher fluorescence signal increases of **2** than of **5** upon protein binding. These data demonstrates quite clearly the advantage of HPBs compared to PBs.

Figure S6. a) Time course of relative fluorescence enhancement for **2** after adding 120 nM human renin; b) time course of fluorescence enhancement for **5** after adding 600 nM human renin. (buffer: 50 mM TRIS, 100 mM NaCl, 1 mM EDTA, 0.1% BSA, pH 7.4, 37 °C, 100 nM **2** and **5** respectively, 120 nM human renin and 600 nM human renin respectively).

b) HPLC analysis to prove proteolytic stability of **2**

Analytical HPLC was performed on a Merck–Hitachi Elite LaChrom chromatograph (column: Varian Polaris C18 A 5 μ 250x46, pore size 220 Å) using A (98.9% H₂O, 1% acetonitrile, 0.1% TFA) and B (98.9% acetonitrile, 1% H₂O, 0.1 % TFA) in a linear gradient (15% A – 80% A in 30 min at 55 °C) with a flow rate of 1 mL/min. Detection of the signals was achieved with a fluorescence detector at λ_{ex} : 667 nm and λ_{em} : 690 nm.

For HPLC analysis the potential reaction of **2** with human renin was quenched by adding TFA (10 μ L). Aliquots of 100 μ L were analyzed by analytical HPLC. Figure S7 shows HPLC traces of **2** without renin and after incubation with renin at 37 °C for 45 min. There were no new signals detectable after 45 min reaction time. It was concluded that the fluorescence enhancement observed upon addition of human renin to **2** is the result of a binding interaction rather than of proteolytic cleavage.

Figure S7. HPLC traces of **2**. green line: HPLC trace of **2** in renin free buffer solution. brown line: HPLC trace of **2** after incubation with renin for 45 min. Conditions: green trace: 100 μ L solution of 100 nM **2** in buffer and addition of 10 Vol% TFA (buffer: 50 mM TRIS, 100 mM NaCl, 1mM EDTA, 0.1% BSA, pH 7.4, 37 °C), brown trace: 100 μ L solution of 100 nM **2** in buffer incubated with 120 nM renin for 45 min at 37°C. After 45 min 10 Vol% TFA was added to stop the reaction (buffer: 50 mM TRIS, 100 mM NaCl, 1mM EDTA, 0.1% BSA, pH 7.4, 37 °C) .

4) Abbreviations

Dabcyl	4-(4- dimethylaminophenylazo)benzoic acid
DMF	<i>N,N</i> -dimethylformamide
Fmoc	9-fluorenylmethoxycarbonyl
HCTU	2-(6-Chloro-1 <i>H</i> -benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate
HPB	Hairpin Peptide Beacon
λ -em	emission wavelength
λ -ex	excitation wavelength
Mmt	4-monomethoxytrityl
NIR	Near-Infrared
NMM	<i>N</i> -methylmorpholine
NMP	<i>N</i> -methyl-2-pyrrolidone
PB	Peptide Beacon
PyBOP	benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TIS	triisopropylsilane

5) Literature

(1) Lynch, B. A.; Loiacono, K. A.; Tiong, C. L.; Adams, S. E.; MacNeil, I. A. *Analytical Biochemistry* **1997**, *247*, 77-82.

(2) PanVera, *Fluorescence Polarization Technical Resource Guide- Analysis of FP Binding Data*. ed.; 2002; Vol. Third Edition, p 8-9.

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