Electronic Supplementary Material (ESI) for Chemical Science.

Cytotoxic peptide-PNA conjugates by RNA-programmed peptidyl transfer with turnover

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Materials and general procedures

Materials: Fmoc/Bhoc-protected PNA monomers were purchased from *Link technologies* (Bellshill, Scotland). Fmoc-protected amino acids were purchased from *Iris Biotech GMBH* (Marktredwitz, Germany). PAGE-purification quality RNA was purchased from *NOXXON Pharma AG* (Berlin, Germany). The resins TentaGel R RAM and TentaGel R RAM high swelling from *RAPP POLYMERE* (Tuebingen, Germany) were used in the synthesis of the peptides and the peptide-PNA conjugates, respectively. HCTU, HBTU and HOBt were purchased from *Chemcube* (Bochum, Germany). DMF and Acetonitrile were purchased from VWR International (Arlington Heights, United States).CellTiter 96® AQueous One Solution Cell Proliferation Assay was purchased from *Promega* (Madison, United States). Water was purified with a Milli-Q Ultra Pure Water Purification System (*Membrapure* Berlin, Germany). Further chemicals were purchased from Sigma-Aldrich (Saint Louis, United States)

Solid-phase synthesis: Peptides and PNA-peptide conjugates were prepared by automated synthesis using the Fmoc solid phase strategy unless specifically noted. The synthesis was performed using 3 mL polyethylene syringe reactors equipped with a fritted disc (*Multisyntech GmbH*, Witten, Germany) in a MultiPep RS parallel synthesizer (*Intavis*, Koeln, Germany) for the case of the peptides and miniscale colums (*Intavis*, Koeln, Germany) in a ResPep-Parallel-Synthesizer (*Intavis,* Koeln, Germany) for the PNA and peptide-PNA conjugates.

Protocol A: The amounts of reagents of the following synthesis protocol correspond to 20 μmol scale of peptides.

Swelling of the resin: The corresponding amount of resin for a 20 μmol scale synthesis was swelling in DMF (500 ⌈L) for 30 min.

Deprotection of the Fmoc-group: DMF/piperidine (4:1, 400 μL) was added to the resin. After 2 min, the procedure was repeated once. The resin was washed (7 × 800 μL DMF). The syringe reactor was vortexed each 3 min for 30 sec.

Coupling of Amino acid monomers: amino acids were coupled by charging the reactor with a solution of the corresponding Fmoc-amino acid (0.6 M solution in DMF, 4 eq.), HBTU (3.6 eq.), HOBt (4 eq.) and NMM (8 eq.). The resulting solution was shaken for 30 min, the resin was filtered and subjected to a recoupling step following the same procedure. The resin was finally washed (7 × 800 μL DMF).

Capping: Ac_2O/2,6-lutidine/DMF (5:6:89, 400 μL) was added and the reaction mixture was shaken each 3 min for 30 sec. After 5 min the resin was washed (7 × 800 μL DMF).

Protocol B: The amounts of reagents of the following synthesis protocol correspond to 5 μmol scale of PNA and PNA-peptide conjugates:

Swelling of the resin: The corresponding amount of resin for a 5 μmol scale synthesis was swelling in DMF (500 ⌈L) for 30 min.

Deprotection of the Fmoc-group: DMF/piperidine (4:1, 2 × 200 μL) was added to the resin for 2 min. The resin was washed (7 × 800 μL DMF).

Coupling of PNA-monomers: PNA-monomers were coupled by charging the reactor with a solution of the corresponding Fmoc-PNA monomer (0.2 M solution in NMP, 4 eq.), HCTU (4 eq.) and NMM (8 eq.). The mixture was shaken for 30 min, the resin was filtered and subjected to a recoupling step following the same procedure. The resin was finally washed (7 × 800 ⌈L DMF).

Coupling of amino acid monomers: amino acids were coupled by charging the reactor with a solution of the corresponding Fmoc-amino acid building block (0.3 M solution in NMP, 4 eq.), HCTU (3.6 eq.), HOBt (3.6 eq.) and NMM (8 eq.). The mixture was shaken for 30 min, the resin was filtered and subjected to a recoupling step following the same procedure. The resin was finally washed (7 × 800 μL DMF).

Capping: Ac_2O/2,6-lutidine/DMF (5:6:89, 200 μL) was added and the reaction mixture. After 5 min the resin was washed (6 × 200 μL DMF).

Final *cleavage- for both protocols (A and B)*: The resin was treated with DMF for 30 min and subsequently washed (10 × 200 μL CH_2Cl_2), dried for 10 min and treated with the cleavage cocktail (40 μL of H_2O, 40 μL TIS, 80 μL Thioanisole and TFA up to 2 mL for 20 mg of resin). The resulting suspension was shaken for 4 h, the resin was filtered off, and the TFA filtrate added to ice-cold Et_2O (10 mL of Et_2O for each mL of TFA). After 10 min, the mixture was centrifuged and the solid was washed with ice-cold Et_2O (20 mL). The solid residue was dried under argon, dissolved in acetonitrile/water (1:1) purified by preparative reverse-phase HPLC.

Purification: The probes were purified by preparative or semipreparative HPLC, performed on an Agilent 1100 series instrument (preparative column: *Nucleodur Gravity C18* A 5μ VP 250/21, pore size 210 Å, flow rate of 13 mL/min; semipreparative column: *Varian Polaris C18* A 5μ 250 × 100, pore size 220 Å, flow rate of 6 mL/min) using eluents A (98.9% H_2O, 1% acetonitrile, 0.1% TFA) and B (98.9% acetonitrile, 1% H_2O, 0.1% TFA) in the corresponding linear gradient. Detection of the signals was achieved with a UV-detector at wavelength 220 and 260 nm for the peptides and PNA conjugates, respectively. The desired products were identified by MALDI-TOF/MS using a Voyager-DE Pro Biospectrometry Workstation (*PerSeptive Biosystems*) and AXIMA Confidence (*Shimadzu Scientific Instruments*). The collected fractions were lyophilized and stored at -20 °C.

Determination of yields: The calculation of yields was based on the concentration of the pure final compounds determined by measurements of the absorbance in Milli-Q water. The peptide molar extinction coefficients at 214 were calculated by using the following equation as reported by Gruppen:[1]

$$\varepsilon_{214\ nm} = 923 \times \#\ \text{peptide bonds} + \sum (\varepsilon_{\text{amino acid}} \times \#\ \text{amino acid})\ M^{-1}cm^{-1}$$

The PNA-conjugate molar extinction coefficients at 260 were calculated using the following equation: [2]

$\varepsilon_{260\,nm} = \{(8.8 \times \#\,T) + (7.3 \times \#\,C) + (11.7 \times \#\,G) + (15.4 \times \#\,A)\} \times 0.9 \times 10^3\ M^{-1}cm^{-1}$

In both equations, # represents the number of peptide bonds or determined amino acid or/and nitrogenous base, depending the case. T represents thymine, C represents cytosine, G represents guanine and A represents adenine.

Characterization of probes: Analytical HPLC was performed on an *Merck Hitachi* serie Elite LaChrom instrument (column: *Varian Polaris C18 A* 5µ 250 × 046, pore size 220 Å). The used eluents were A (98.9% H_2O, 1% acetonitrile, 0.1% TFA) and B (98.9% acetonitrile, 1% H_2O, 0.1% TFA) in the corresponding gradient with a flow rate of 1 mL/min. Electrospray Ionization Mass Spectrometry (ESI-MS) was performed by direct injection on an Agilent 1100 series HPLC/MS instrument with a VL quadrupole mass spectrometer, model in positive scan mode. MALDI-TOF/MS were also recorded (matrixes: DHL and sinapinic acid).

Synthesis and characterization data of the peptides

The synthesis of the peptides was performed following the protocol A. The peptides were purified by single preparative HPLC using the linear gradient 3% B to 30% B in 30 min. The characterization of the probes were carried out by recording the MALDI-TOF/MS and the analytical HPLC chromatogram using the using the linear gradient 3% B to 50% B in 20 min.

1: R_7G_2KLAKLAKKLAKLAK; 27.0 mg; 49 % yield; t_R = 14.9. Formula: $C_{120}H_{231}N_{51}O_{24}$. MALDI-TOF/MS: $[M+H]^+$ found = 2771.8, ($[M+H]^+$ calcd. = 2771.8501).

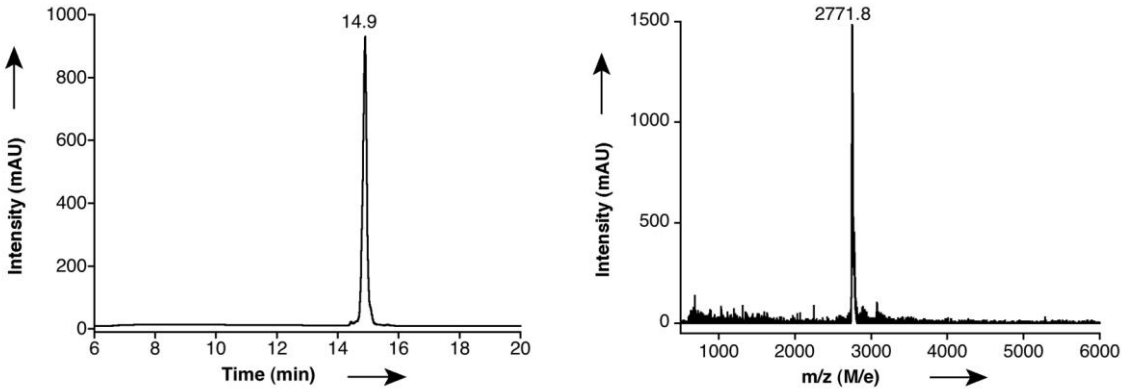


Figure S1. A) HPLC and B) MALDI-TOF MS analysis of purified compound **1**.

2: R_7G_2KCAKLAKKLAKLAK; 23.8 mg; 43 % yield; t_R = 13.3. Formula: $C_{117}H_{225}N_{51}O_{24}S$. MALDI-TOF/MS: $[M+H]^+$ found = 2761.5, ($[M+H]^+$ calcd. = 2761.7753).

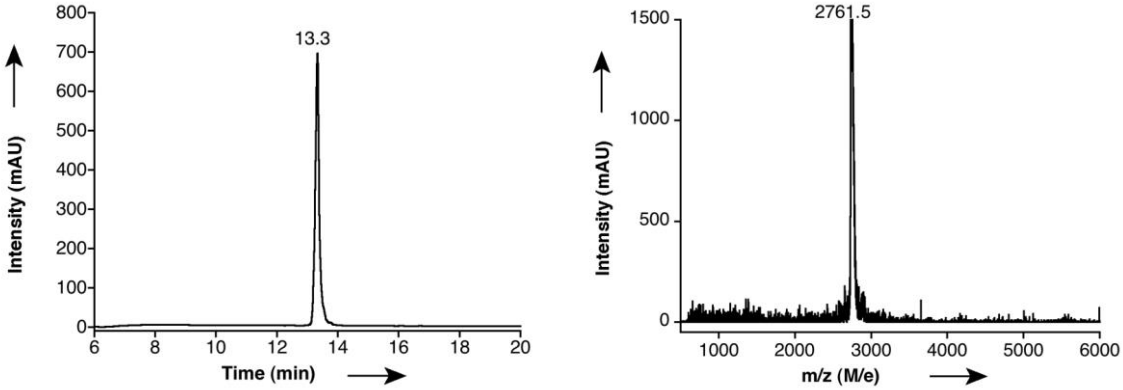


Figure S2. A) HPLC and B) MALDI-TOF MS analysis of purified compound **2**.

3: $R_7G_2KLCKLAKKLAKLAK$; 23.0 mg; 41 % yield; t_R = 14.5. Formula: $C_{120}H_{231}N_{51}O_{24}S$. MALDI-TOF/MS: $[M+H]^+$ found = 2804.0, ($[M+H]^+$ calcd. = 2803.8222).

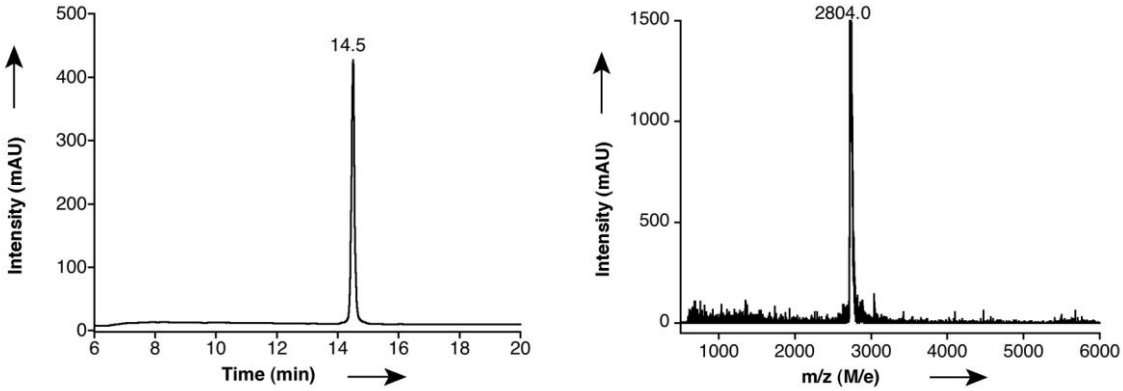


Figure S3. A) HPLC and B) MALDI-TOF MS analysis of purified compound **3**.

4: $R_7G_2KLACLAKKLAKLAK$; 26.9 mg; 49 % yield; t_R = 15.3. Formula: $C_{117}H_{224}N_{50}O_{24}S$. MALDI-TOF/MS: $[M+H]^+$ found = 2747.0, ($[M+H]^+$ calcd. = 2746.7644).

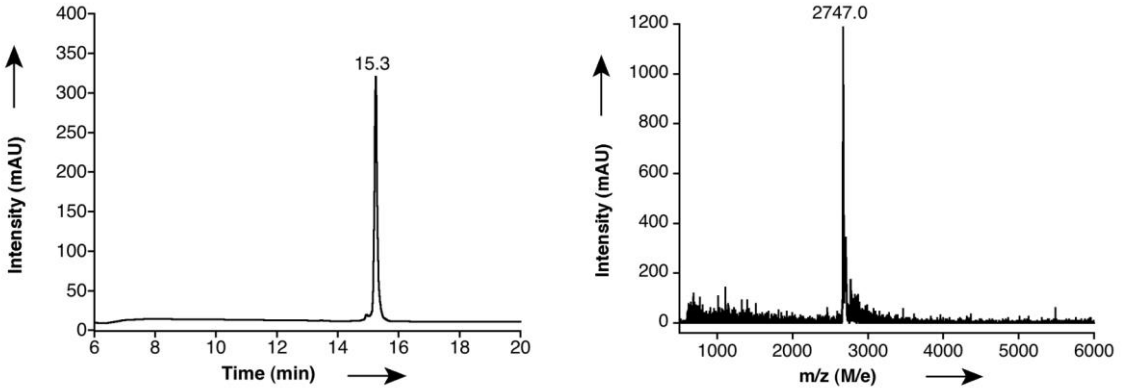


Figure S4. A) HPLC and B) MALDI-TOF MS analysis of purified compound **4**.

5: $R_7G_2KLAKCAKKLAKLAK$; 23.5 mg; 43 % yield; t_R = 13.3. Formula: $C_{117}H_{225}N_{51}O_{24}S$. MALDI-TOF/MS: $[M+H]^+$ found = 2761.9, ($[M+H]^+$ calcd. = 2761.7753).

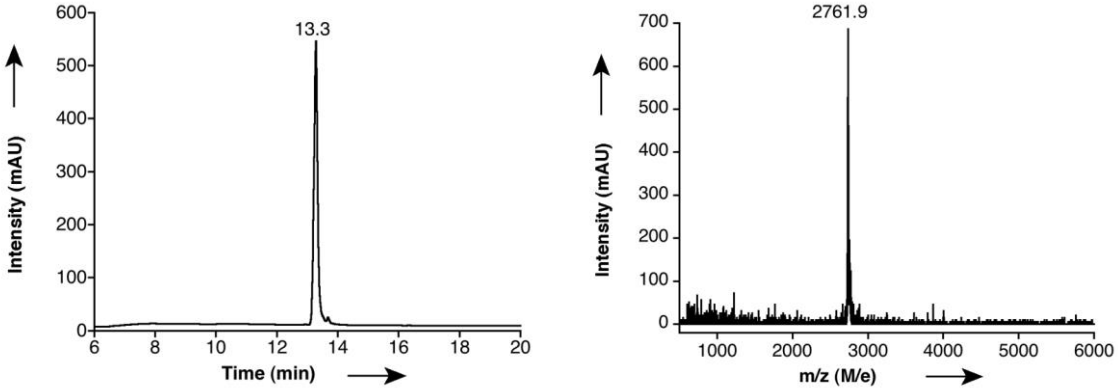


Figure S5. A) HPLC and B) MALDI-TOF MS analysis of purified compound **5**.

6: $R_7G_2KLAKLCKKLAKLAK$; 25.8 mg; 46 % yield; t_R = 15.1. Formula: $C_{120}H_{231}N_{51}O_{24}S$. MALDI-TOF/MS: $[M+H]^+$ found = 2802.8, ($[M+H]^+$ calcd. = 2803.8222).

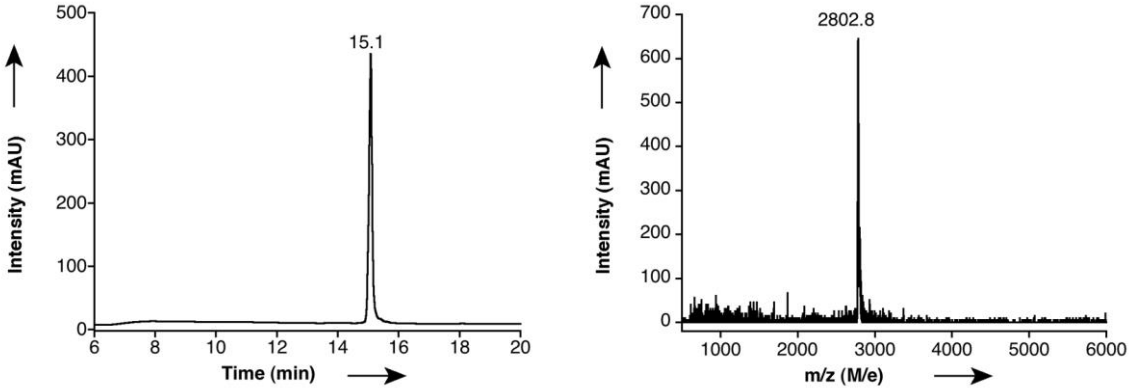


Figure S6. A) HPLC and B) MALDI-TOF MS analysis of purified compound **6**.

7: $R_7G_2KLAKLACKLAKLAK$; 28.0 mg; 51 % yield; t_R = 15.8. Formula: $C_{117}H_{224}N_{50}O_{24}S$. MALDI-TOF/MS: $[M+H]^+$ found =2743.4, ($[M+H]^+$ calcd. = 2746.7644).

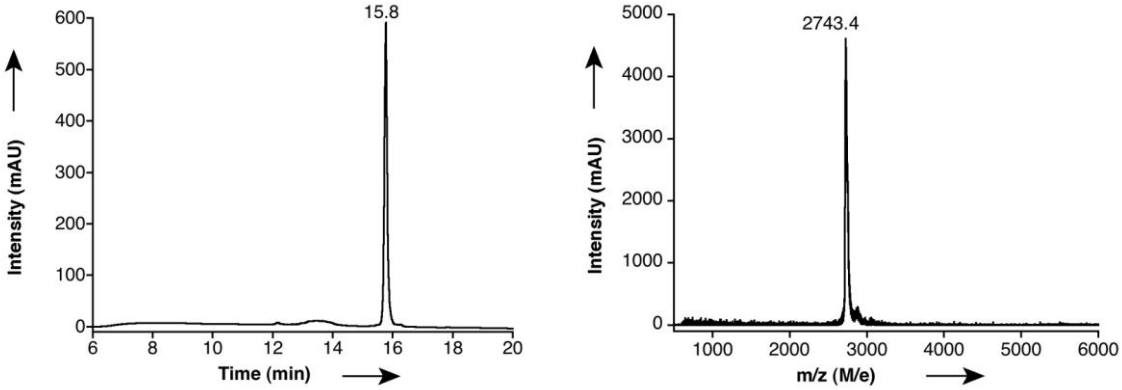


Figure S7. A) HPLC and B) MALDI-TOF MS analysis of purified compound **7**.

8: $R_7G_2KLAKLAKCLAKLAK$; 23.6 mg; 43 % yield; t_R = 16.2. Formula: $C_{117}H_{224}N_{50}O_{24}S$. MALDI-TOF/MS: $[M+H]^+$ found = 2746.7, ($[M+H]^+$ calcd. = 2746.7644).

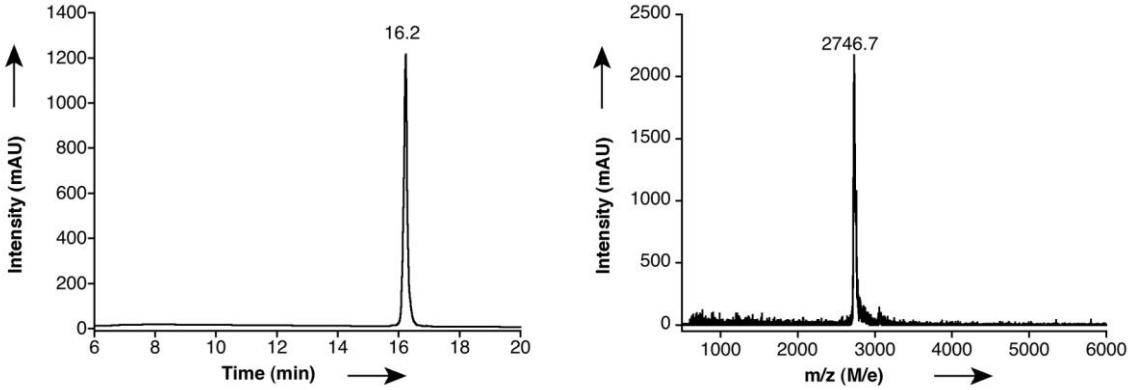


Figure S8. A) HPLC and B) MALDI-TOF MS analysis of purified compound **8**.

9: R_7G_2KLAKLAKKCAKLAK; 26.5 mg; 48 % yield; t_R = 13.2. Formula: $C_{117}H_{225}N_{51}O_{24}S$. MALDI-TOF/MS: $[M+H]^+$ found = 2761.7, ($[M+H]^+$ calcd. = 2761.7753).

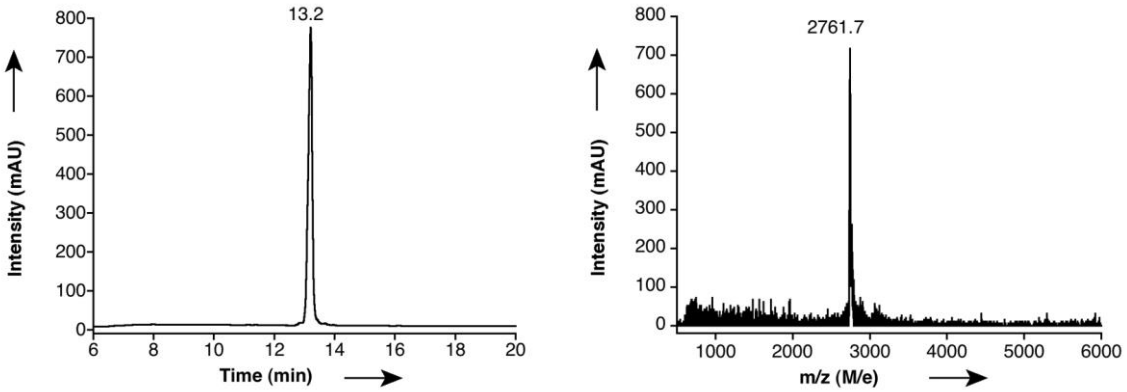


Figure S9. A) HPLC and B) MALDI-TOF MS analysis of purified compound **9**.

10: R_7G_2CAKLAKKLAKLAK; 27.4 mg; 52 % yield; t_R = 14.2. Formula: $C_{111}H_{213}N_{49}O_{23}S$. MALDI-TOF/MS: $[M+H]^+$ found = 2632.9, ($[M+H]^+$ calcd. = 2633.6803).

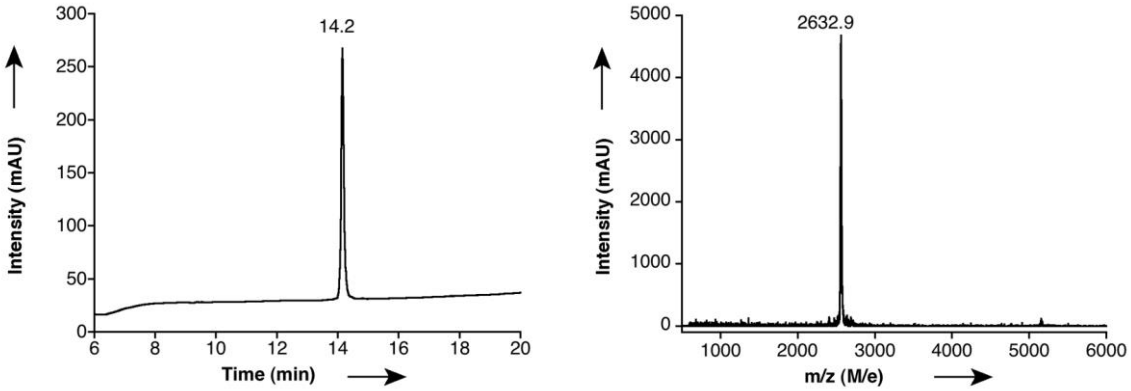


Figure S10. A) HPLC and B) MALDI-TOF MS analysis of purified compound **10**.

11: R_7G_2CKLAKKLAKLAK; 32.3 mg; 63 % yield; t_R = 13.2. Formula: $C_{108}H_{208}N_{48}O_{22}S$. MALDI-TOF/MS: $[M+H]^+$ found = 2562.6, ($[M+H]^+$ calcd. = 2562.6432).

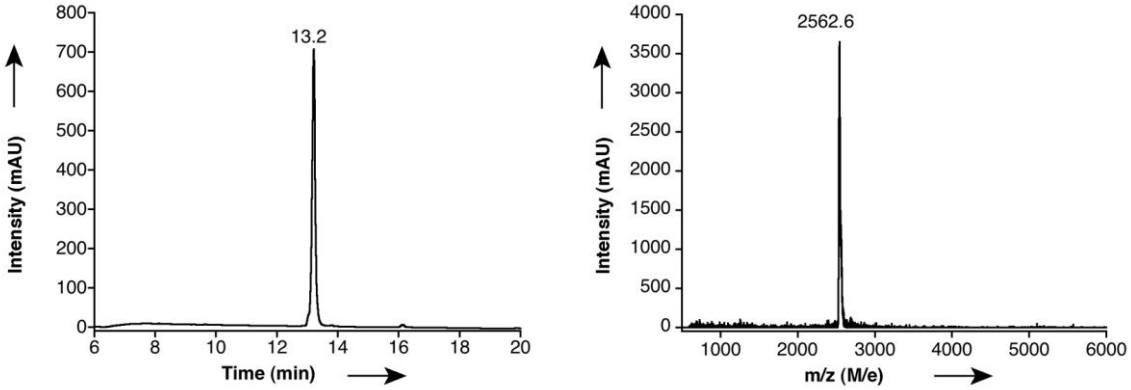


Figure S11. A) HPLC and B) MALDI-TOF MS analysis of purified compound **11**.

12: $R_7G_2CLAKKLAKLAK$; 35.0 mg; 72 % yield; t_R = 13.9. Formula: $C_{102}H_{196}N_{46}O_{21}S$. MALDI-TOF/MS: $[M+H]^+$ found = 2434.6, ($[M+H]^+$ calcd. = 2434.5482).

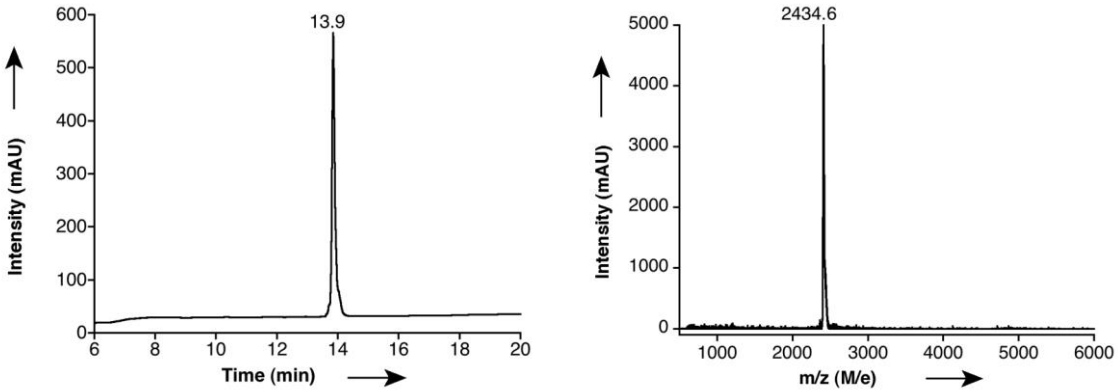


Figure S12. A) HPLC and B) MALDI-TOF MS analysis of purified compound **12**

13: $R_7G_2CAKKLAKLAK$; 30.2 mg; 65 % yield; t_R = 12.0. Formula: $C_{96}H_{185}N_{45}O_{20}S$. MALDI-TOF/MS: $[M+H]^+$ found = 2321.5, ($[M+H]^+$ calcd. = 2321.4641).

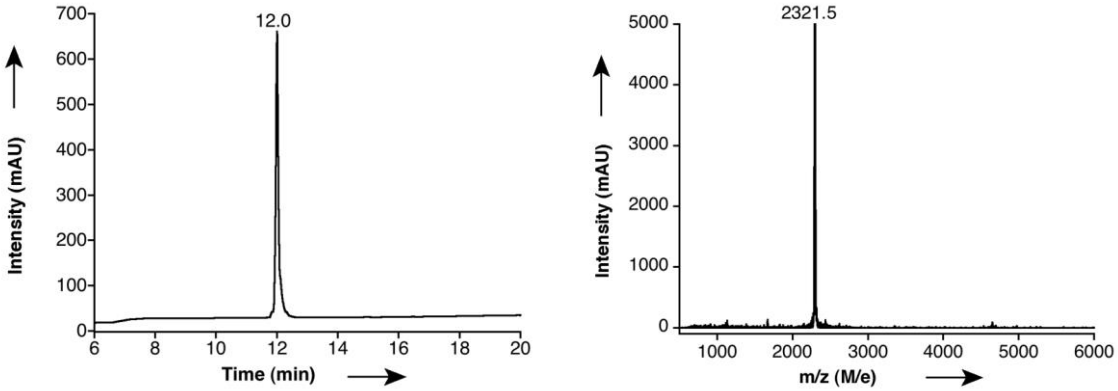


Figure S13. A) HPLC and B) MALDI-TOF MS analysis of purified compound **13**.

14: $R_7G_2CKKLAKLAK$; 27.0 mg; 60 % yield; t_R = 11.2. Formula: $C_{93}H_{180}N_{44}O_{19}S$. MALDI-TOF/MS: $[M+H]^+$ found = 2249.5, ($[M+H]^+$ calcd. = 2250.4270).

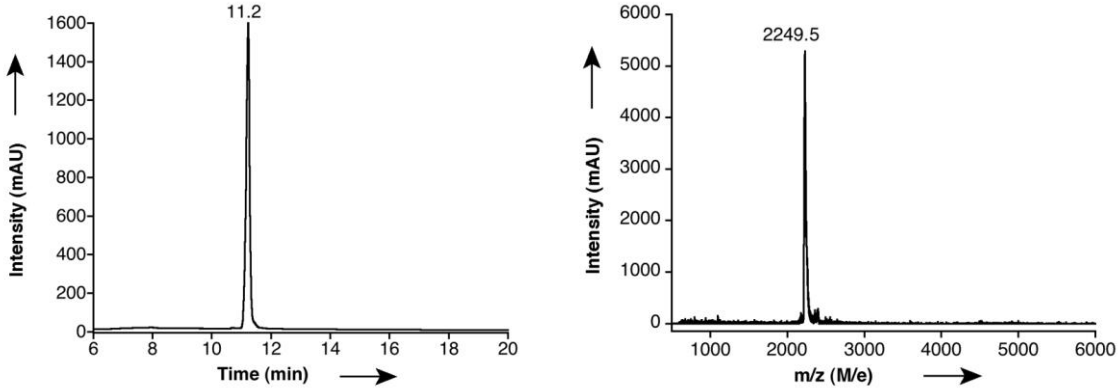


Figure S14. A) HPLC and B) MALDI-TOF MS analysis of purified compound **14**.

15: $R_7G_2CKLAKLAK$; 28.0 mg; 66 % yield; t_R = 11.7. Formula: $C_{87}H_{168}N_{42}O_{18}S$. MALDI-TOF/MS: $[M+H]^+$ found = 2120.8, ($[M+H]^+$ calcd. = 2122.3242).

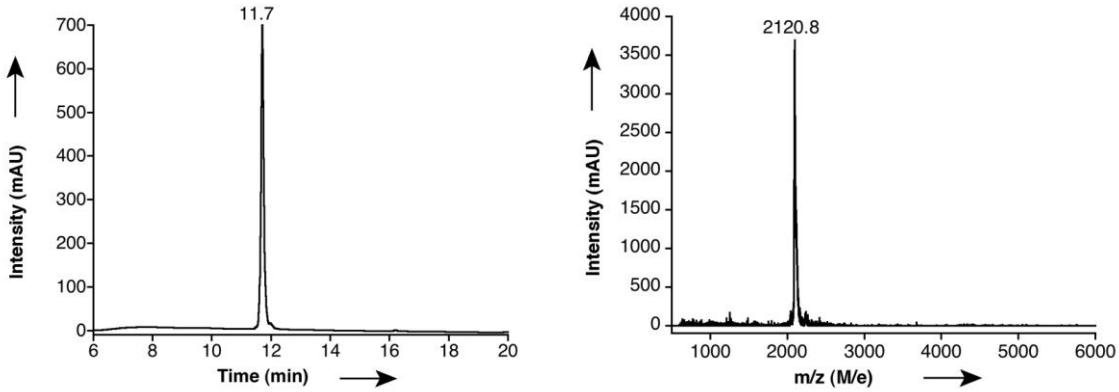


Figure S15. A) HPLC and B) MALDI-TOF MS analysis of purified compound **15**.

16: $R_7G_2CLAKLAK$; 29.1 mg; 73 % yield; t_R = 12.3. Formula: $C_{81}H_{156}N_{40}O_{17}S$. MALDI-TOF/MS: $[M+H]^+$ found = 1995.6, ($[M+H]^+$ calcd. = 1994.2371).

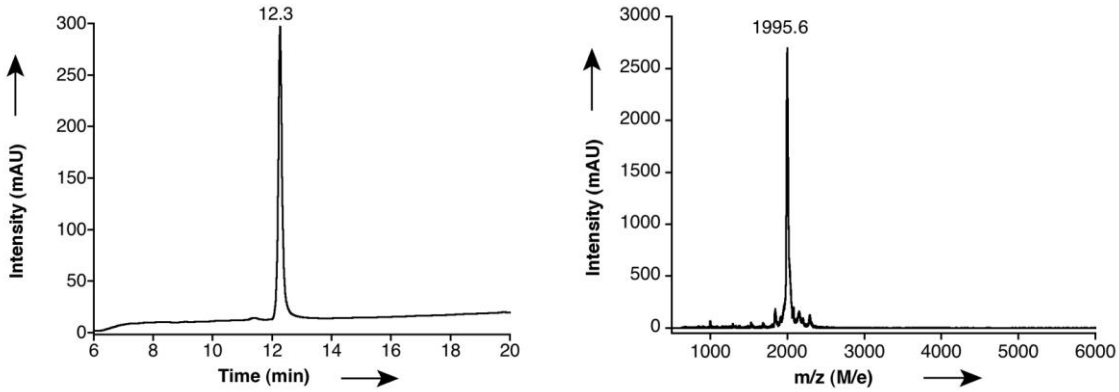


Figure S16. A) HPLC and B) MALDI-TOF MS analysis of purified compound **16**.

17: $R_7G_2CAKLAK$; 31.2 mg; 83 % yield; t_R = 11.0. Formula: $C_{75}H_{145}N_{39}O_{16}S$. MALDI-TOF/MS: $[M+H]^+$ found = 1880.1, ($[M+H]^+$ calcd. = 1881.1530).

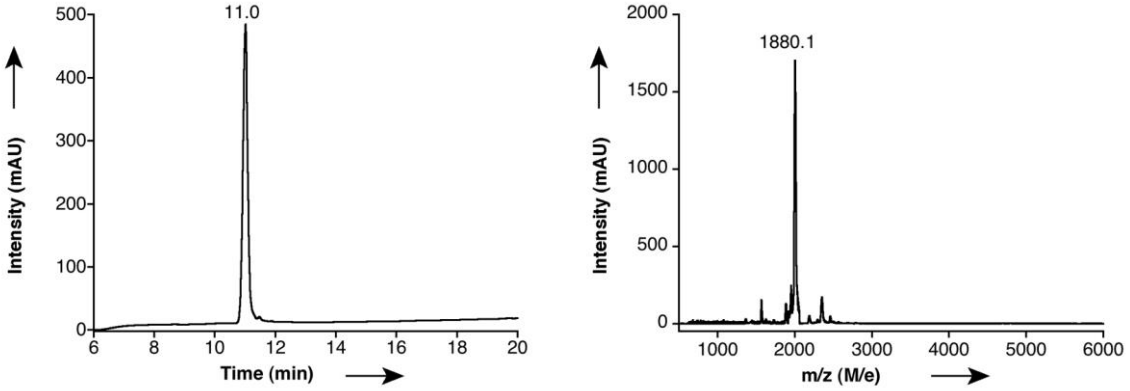


Figure S17. A) HPLC and B) MALDI-TOF MS analysis of purified compound **17**.

18: KLAKLAKKLAKLAK; 25.6 mg; 82 % yield; t_R = 16.9. Formula: $C_{74}H_{141}N_{21}O_{15}$. MALDI-TOF/MS: $[M+H]^+$ found = 1564.9, ($[M+H]^+$ calcd. = 1565.0994).

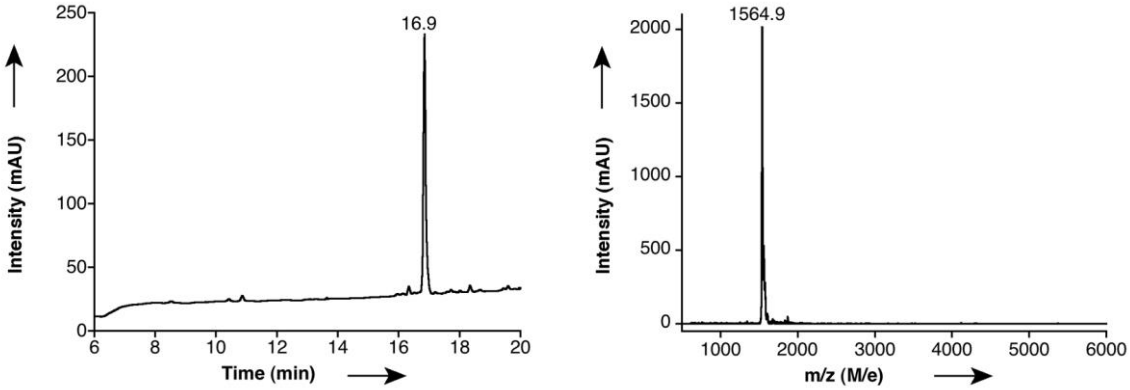


Figure S18. A) HPLC and B) MALDI-TOF MS analysis of purified compound **18**.

19: R_7G_2; 20.8 mg; 82 % yield; t_R = 7.7 Formula: $C_{48}H_{95}N_{31}O_{10}$. MALDI-TOF/MS: $[M+H]^+$ found = 1266.8, ($[M+H]^+$ calcd. = 1266.7956).

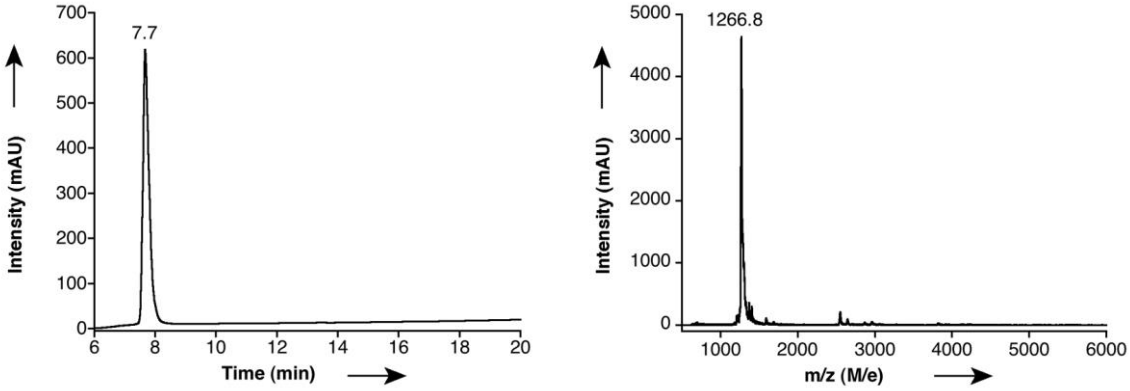


Figure S19. A) HPLC and B) MALDI-TOF MS analysis of purified compound **19**.

Synthesis of the PNA conjugates

The synthesis of the PNA conjugates was mainly performed by means of protocol B. For characterization MALDI-TOF/MS and analytical HPLC profiles (linear gradient 3% B to 50% B in 30 min unless specifically noted) were measured.

Synthesis and characterization data of the PNA conjugates used for the optimization of the RNA catalytic transfer reaction

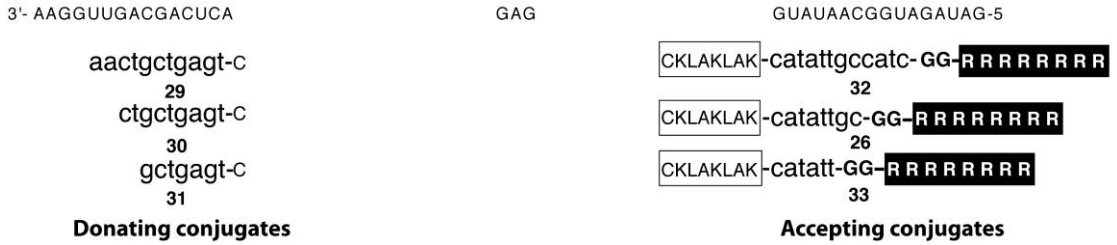


Figure S20. Illustration of the different PNA pairs synthesized and studied for the optimization of the length of the PNA probes.

29: OD_{260} = 66.0; 12 % yield; t_R = 13.3. Formula: $C_{124}H_{156}N_{66}O_{35}S$. MALDI-TOF/MS: $[M+H]^+$ found = 3162.3, ($[M+H]^+$ calcd. = 3162.2255).

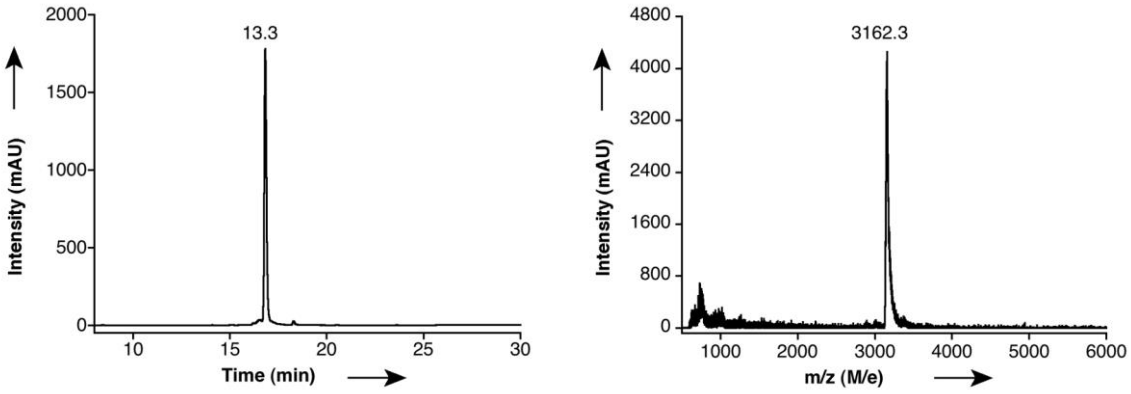


Figure S21. A) HPLC and B) MALDI-TOF MS analysis of purified compound **29**.

30: OD_{260} = 65.9; 16 % yield; t_R = 16.8. Formula: $C_{102}H_{130}N_{52}O_{31}S$. MALDI-TOF/MS: $[M+H]^+$ found = 2611.9, ($[M+H]^+$ calcd. = 2611.9994).

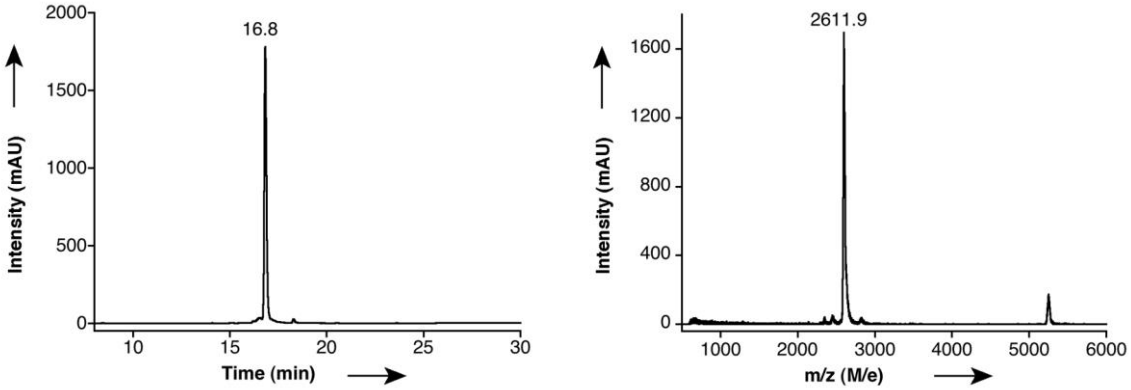


Figure S22. A) HPLC and B) MALDI-TOF MS analysis of purified compound **30**.

31: OD_{260} = 61.1; 18 % yield; t_R = 13.6. Formula: $C_{81}H_{103}N_{43}O_{24}S$. MALDI-TOF/MS: $[M+H]^+$ found = 2094.8, ($[M+H]^+$ calcd. = 2094.7960).

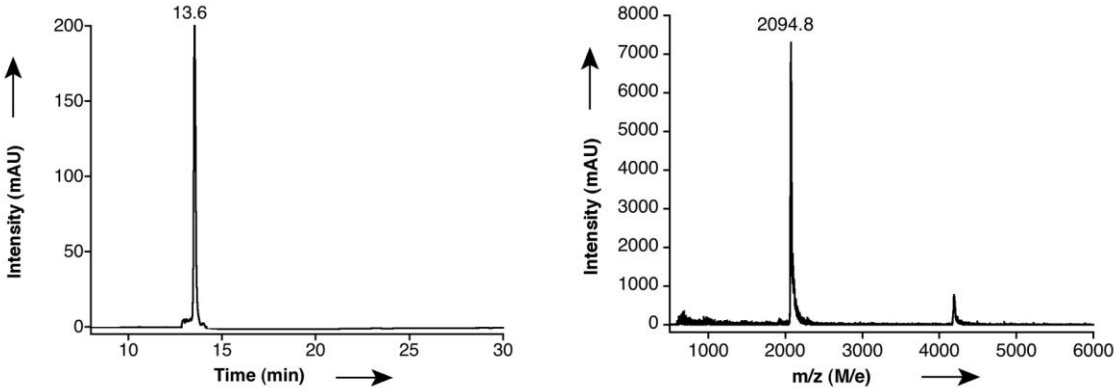


Figure S23. A) HPLC and B) MALDI-TOF MS analysis of purified compound **31**.

32: OD_{260} = 44.0; 8 % yield; t_R = 15.1. Formula: $C_{219}H_{338}N_{110}O_{55}S$. MALDI-TOF/MS: $[M+H]^+$ found = 5421.6, ($[M+H]^+$ calcd. = 5421.6832)

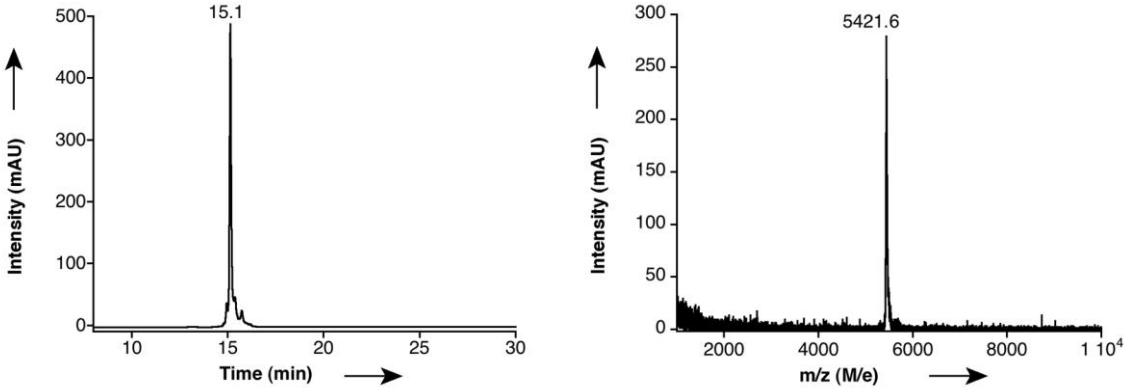


Figure S24. A) HPLC and B) MALDI-TOF MS analysis of purified compound **32**.

26: OD_{260} = 41.3; 11 % yield; t_R = 14.9. Formula: $C_{177}H_{285}N_{89}O_{43}S$. MALDI-TOF/MS: $[M+H]^+$ found = 4376.8, ($[M+H]^+$ calcd. = 4378.2649).

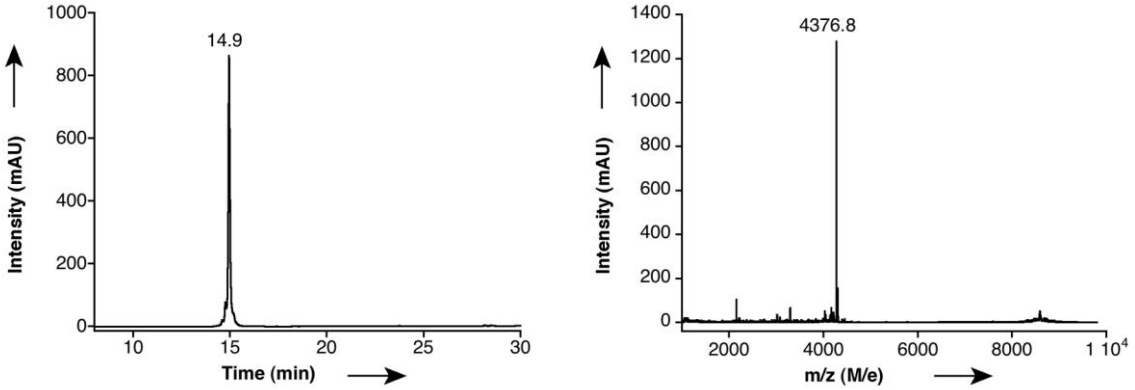


Figure S25. A) HPLC and B) MALDI-TOF MS analysis of purified compound **26**.

33: OD_{260} = 20.3; 7 % yield; t_R = 16.6. Formula: $C_{156}H_{259}N_{77}O_{37}S$. MALDI-TOF/MS: $[M+H]^+$ found = 3834.8, ($[M+H]^+$ calcd. = 3836.0551).

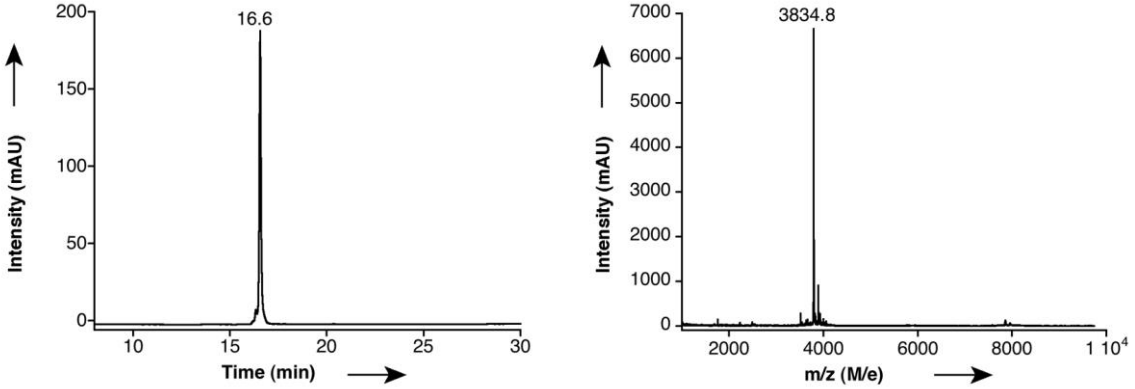
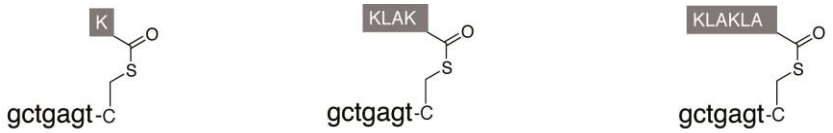


Figure S26. A) HPLC and B) MALDI-TOF MS analysis of purified compound **33**.

Synthesis and characterization data of the peptide-PNA donors used for the optimization of the RNA catalytic transfer reaction



20 **22** **24**

Figure S27. Structures of the different donating PNA probes synthesized and studied.

The thioester-linked peptide-PNA conjugates **20** and **22** were synthesized by a convergent strategy (Figure S28). This involved a thiol exchange reaction between a PNA-Cys conjugate, which was readily accessible via Fmoc-based solid phase synthesis, and a peptide thioester prepared by manual synthesis at the 4-sulfamylbutyryl resin (*Novabiochem*).

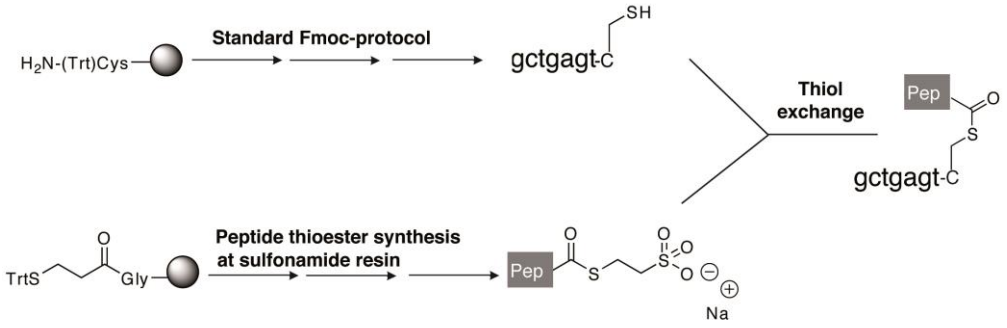


Figure S28. Scheme of the convergent strategy.

Peptide thioester synthesis at the sulfonamide resin:

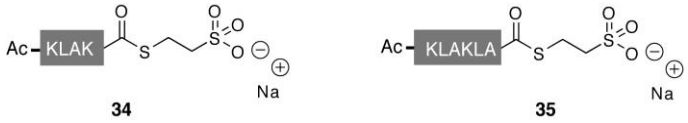


Figure S29. Structures of the different peptide thioester synthesized at the sulfonamide resin.

Swelling and preloading of the resin: The corresponding amount of resin for a 20 μmol scale synthesis was allowed to swell in $CHCl_3$ (500 μL) for 30 min. The resin loading (ca. 0.2 mmol/g) was performed with the corresponding Fmoc-protected amino acid (0.2 M solution in $CHCl_3$, 4 eq.), PyBOP (4 eq.) and DIPEA (12 eq.). The mixture was shaken for 45 min, the resin was filtered and subjected to a recoupling step following the same procedure. The resin was finally washed (5 × 1 mL DMF, 5 × 1 mL CH_2Cl_2, 5 × 1 mL DMF)

Deprotection of the Fmoc-group: DMF/piperidine (4:1, 2 × 500 μL × 5 min) was added to the resin. Afterwards, the resin was washed (5 × 1 mL DMF, 5 × 1 mL CH_2Cl_2, 5 × 1 mL DMF).

Coupling of Amino acid monomers: amino acids were coupled by charging the reactor with a solution of the corresponding Fmoc-amino acid (0.2 M solution in DMF, 4 eq.), HBTU (3.6 eq.), HOBt (4 eq.) and DIPEA (8 eq.). The resulting solution was shaken for 30 min and then the resin was washed (5 × 1 mL DMF, 5 × 1 mL CH_2Cl_2, 5 × 1 mL DMF).

Capping: Ac_2O/2,6-lutidine/DMF (5:6:89, 1 mL) was added and the reaction mixture was shaken for 5 min and then the resin was washed (5 × 1 mL DMF, 5 × 1 mL CH_2Cl_2, 5 × 1 mL DMF).

Activation and thiolysis of acyl-sulfonamide bond:[3] The resin was washed (5 × 1 mL DMF, 5 × 1 mL CH_2Cl_2, 6 × 1 mL THF). A solution of THF/(Trimethylsilyl)diazomethane (2M) in hexene (1:1 400 μL) was added to the resin. The mixture was agitated for 1 h. The resin was washed (5 × 1 mL DMF, 5 × 1 mL CH_2Cl_2, 5 × 1 mL DMF) and subsequently the thiolysis was performed by adding to the active resin a solution of benzyl mercaptan (50 eq. 117 μL) and sodium benzenethiolate (3 eq. 7.9 mg) in DMF (900 μL) for 48 h. The supernatant was filtrated, combined with the washing of the resin (3 × 500 μL DMF) and concentrated to dryness.

Deprotection and purification: The isolated crude peptide was treated with a mixture of TFA/H_2O/TIS (96:2:2, 2.5 mL, 2 h). The compound precipitated upon addition of ice-cold Et_2O (10 mL of Et_2O for each mL of TFA). The crude product was dried under argon and dissolved in 50 mM MeSNa, 200 mM phosphate buffer pH=7, 20 mM TCEP. The solution was agitated for 1 h and purified by preparative reverse-phase HPLC using the linear gradient 0% B to 30% B in 30 min. The purified peptide thioesters were characterized by measurements of ESI/MS spectra and HPLC analysis (linear gradient 0% B to 30% B in 30 min).

34: 4.1 mg; 33 % yield; t_R = 15.0. Formula: $C_{25}H_{47}N_6O_8S_2$. MALDI-TOF/MS: $[M+H]^+$ found = 624.2, ($[M+H]^+$ calcd. = 624.2975).

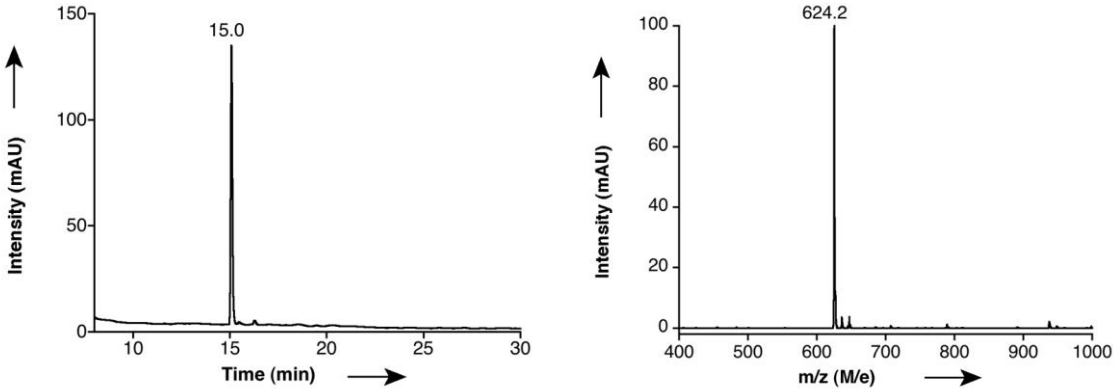


Figure S30. A) HPLC and B) ESI/MS analysis of purified compound **34**.

35: 4.5 mg; 28 % yield; t_R = 24.6. Formula: $C_{34}H_{63}N_8O_{10}S_2$. MALDI-TOF/MS: $[M+H]^+$ found = 808.4, ($[M+H]^+$ calcd. = 808.4187).

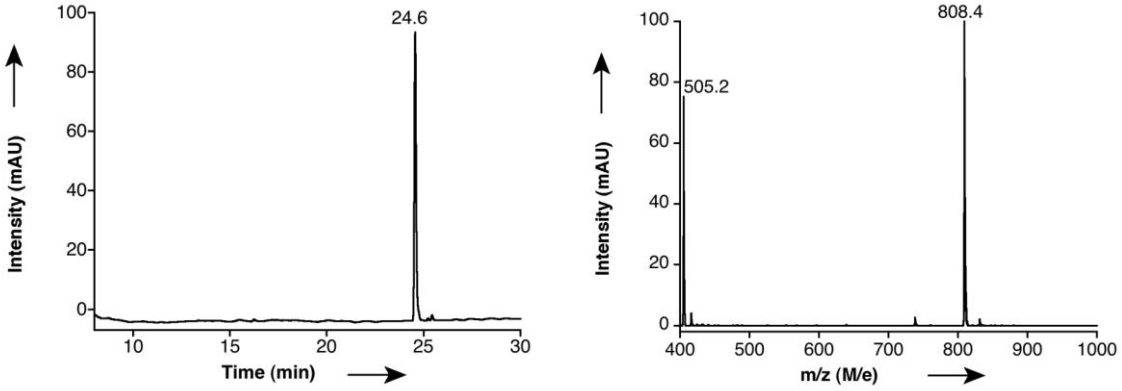


Figure S31. A) HPLC and B) ESI/MS analysis of purified compound **35**.

Thiol exchange reaction: The cysteinyl-PNA conjugate **31** (1.6 μmol, 1.5 eq.) was dissolved in a degassed 6 mL solution comprised of 100 mM phosphate buffer pH7, 20mM TCEP/CH_3CN (4:1). After 30 min, the corresponding peptide-thioester was added (1.1 μmol, 1 eq.) and the solution was stirred at ambient

temperature for 6 h, after which the crude was purified by semipreparative HPLC and characterized by MALDI-TOF mass spectrometry.

22: OD_{260} = 27.1; 8 % yield; t_R = 17.7. Formula: $C_{113}H_{161}N_{51}O_{30}S$. MALDI-TOF/MS: $[M+H]^+$ found = 2745.2, ($[M+H]^+$ calcd. = 2745.2439).

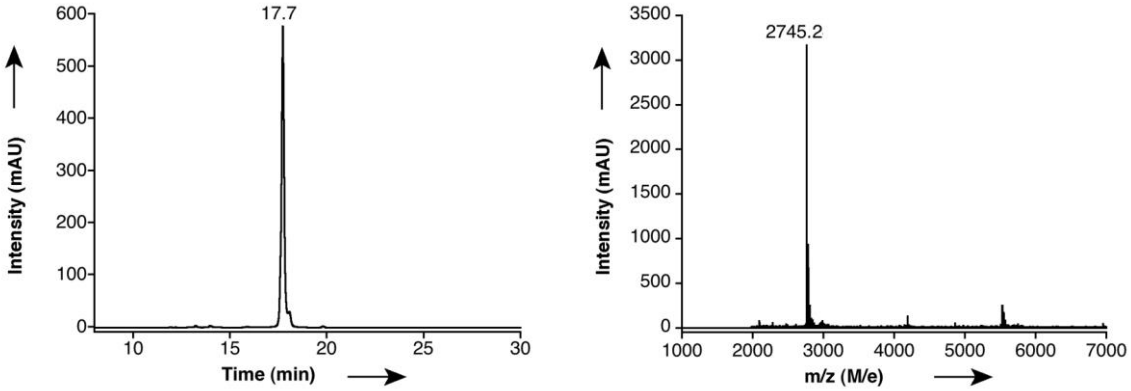


Figure S32. A) HPLC and B) MALDI-TOF MS analysis of purified compound **22**.

24: OD_{260} = 30.5; 9 % yield; t_R = 16.5 (linear gradient 0% B to 30% B in 30 min). Formula: $C_{125}H_{172}N_{58}O_{36}S$. MALDI-TOF/MS: $[M+H]^+$ found = 3094.2, ($[M+H]^+$ calcd. = 3094.3210).

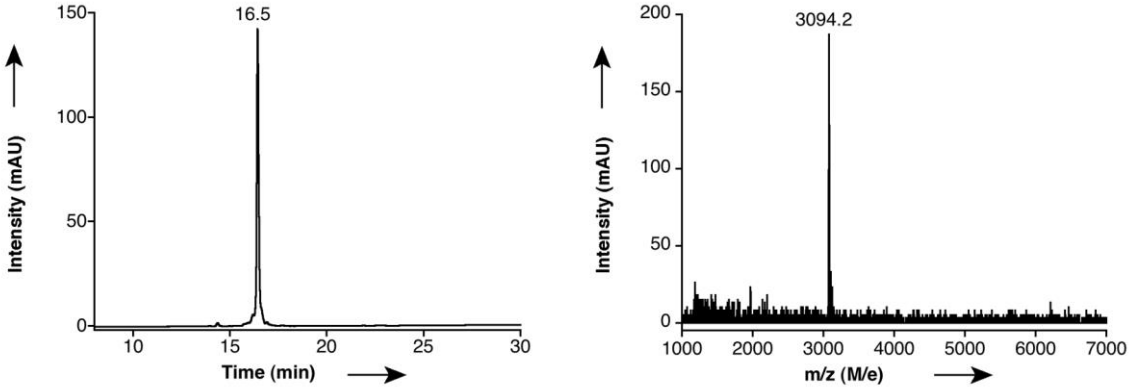


Figure S33. A) HPLC and B) MALDI-TOF MS analysis of purified compound **24**.

The synthesis of **20** was carried out by using Boc/Z-protected building blocks.

Swelling and preloading of the resin: the amount of MBHA resin required for a 5 μmol scale synthesis was allowed to swell in DMF (500 μL) for 30 min. Then the resin was loaded with Boc-Cys(Fm)-OH according to standard protocols (ca. 0.2 mmol/g) (see: Novabiochem catalog 2010/2011)

Deprotection of the Boc-group: addition of TFA/m-cresol (95:5, 5 min, 1 mL) was followed by washing (10 × 1 mL CH_2Cl_2, 5 × 1 mL DMF) of the resin.

Coupling of PNA monomers: PNA monomers were coupled by charging the reactor with a solution of the corresponding Boc-PNA monomer (0.2 M solution in DMF, 4 eq.), PyBOP (4 eq.) and NMM (8 eq.). After 30 min, the resin was washed (5 × 1 mL DMF, 10 × 1 mL CH_2Cl_2).

Capping: Ac_2O/2,6-lutidine/DMF (5:6:89, 400 µL) was added and the reaction mixture was shaken for 5 min. Afterwards the resin was washed (5 × 1 mL DMF, 10 × 1 mL CH_2Cl_2).

Deprotection of the Fm-group and coupling of lysine: A solution of piperidine/DBU/2-mercaptoethanol/degassed DMF (20:2:2, 2 × 5 min, 1 mL) was added to the resin. Afterwards the resin was washed (5 × 1 mL DMF, 10 × 1 mL CH_2Cl_2) and the Boc/Boc-protected lysine monomer was coupled in the same way as it was explained before.

Final cleavage from the solid support: the resin was treated with a mixture of TFA/TFMSA/m-cresol (85:5:5, 2 mL) for 2 h and subsequently washed with 2x 100 µL TFA. The resin was filtered off, and the TFA filtrate added to ice-cold Et_2O (10 mL of Et_2O for each mL of TFA). After 10 min, the mixture was centrifuged and the solid was washed with ice-cold Et_2O (20 mL). The solid residue was dried under argon, dissolved in acetonitrile/water (1:1) purified by semipreparative reverse-phase HPLC.

20: OD_{260} = 27.1; 8 % yield; t_R = 12.6. Formula: $C_{89}H_{117}N_{45}O_{25}S$. MALDI-TOF/MS: $[M+H]^+$ found = 2250.0, ($[M+H]^+$ calcd. = 2248.91).

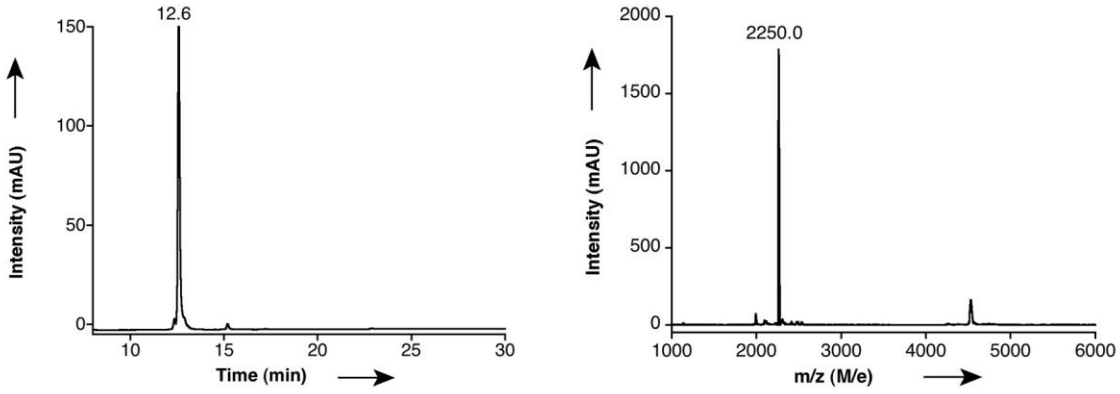


Figure S34. A) HPLC and B) MALDI-TOF MS analysis of purified compound **20**.

Synthesis and characterization data of the peptide-PNA acceptors used for the optimization of the RNA catalytic transfer reaction

CAKLAKKLAKLAK-catattgccatc-G-$CONH_2$

21

CAKKLAKLAK-catattgccatc-G-$CONH_2$

23

CKLAKLAK-catattgccatc-G-$CONH_2$

25

Figure S35. Structures of the different accepting PNA probes synthesized and studied.

The synthesis of the petide-PNA acceptors was performed following the protocol B. The compounds were purified by semipreparative HPLC using the linear gradient 3% B to 30% B in 30 min. The characterization of the probes was carried out by measrements of MALDI-TOF mass spectra and HPLC analysis (linear gradient 3% B to 50% B in 30 min).

21: OD_{260} = 27.5; 5 % yield; t_R = 17.4. Formula: $C_{193}H_{284}N_{84}O_{51}S$. MALDI-TOF/MS: $[M+H]^+$ found = 4628.5, ($[M+H]^+$ calcd. = 4627.2011).

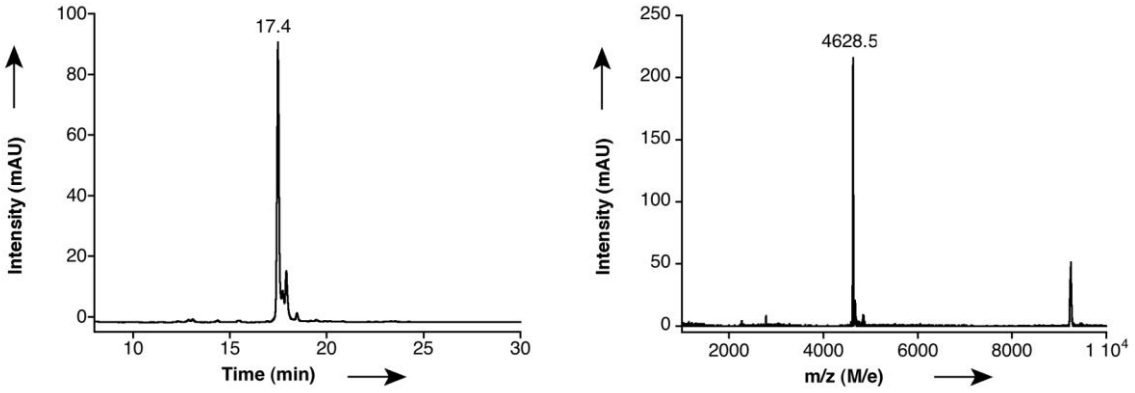


Figure S36. A) HPLC chromatogram of the compound **21** after purification. B) MALDI-TOF spectrum of the compund **21**.

23: OD_{260} = 44.0; 8 % yield; t_R = 20.4 (linear gradient 0% B to 30% B in 30 min). Formula: $C_{178}H_{256}N_{80}O_{48}S$. MALDI-TOF/MS: $[M+H]^+$ found = 4315.0, ($[M+H]^+$ calcd. = 4314.9849).

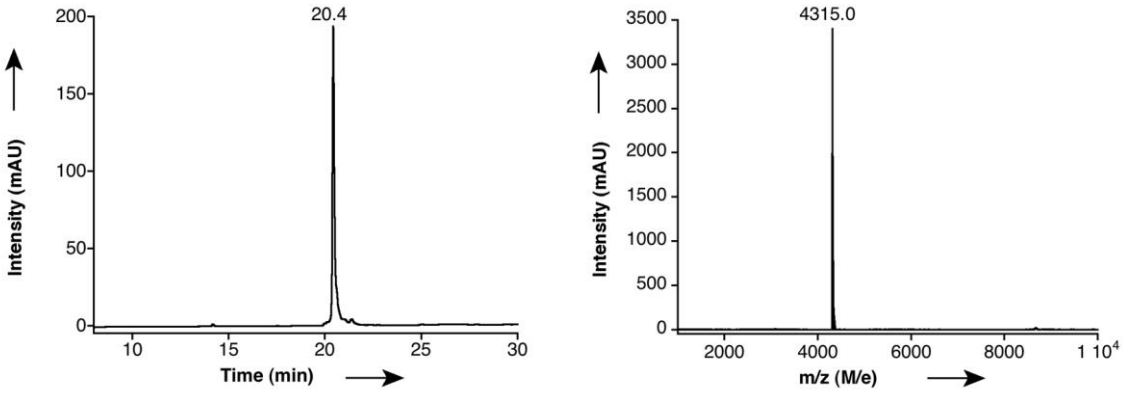


Figure S37. A) HPLC chromatogram of the compound **23** after purification. B) MALDI-TOF spectrum of the compund **23**.

25: OD_{260} = 82.5; 15 % yield; t_R = 15.2. Formula: $C_{169}H_{239}N_{77}O_{46}S$. MALDI-TOF/MS: $[M+H]^+$ found = 4114.9, ($[M+H]^+$ calcd. = 4115.8528).

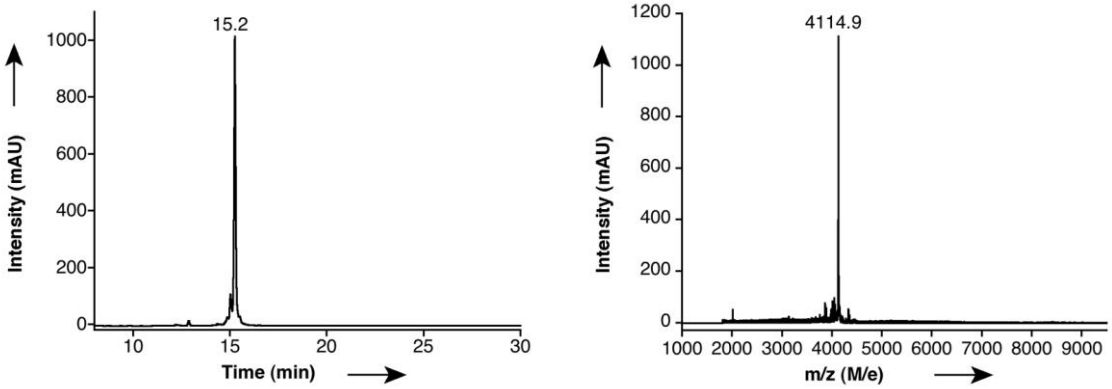


Figure S38. A) HPLC chromatogram of the compound **25** after purification. B) MALDI-TOF spectrum of the compund **25**.

Synthesis and characterization data of the full length peptide-PNA product as authentic reference

36

Figure S39. Structure of the desired peptide-PNA product **36**.

The synthesis of the peptide-PNA conjugate **36** was performed following the protocol B. The compound was purified by semipreparative HPLC using the linear gradient 3% B to 50% B in 30 min. The characterization of the probes were carried out by a measurement of the MALDI-TOF mass spectrum and HPLC analysis (linear gradient 3% B to 50% B in 30 min).

36: OD_{260} = 55.0; 10 % yield; t_R = 22.7. Formula: $C_{209}H_{343}N_{97}O_{50}S$. MALDI-TOF/MS: $[M+H]^+$ found = 5044.0, ($[M+H]^+$ calcd. = 5044.7078).

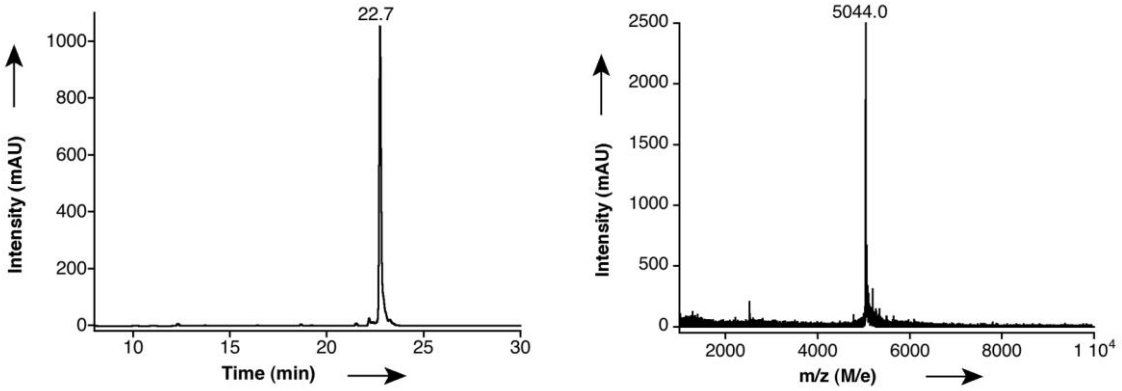


Figure S40. A) HPLC chromatogram of the compound **36** after purification. B) MALDI-TOF spectrum of the compund **36**.

RNA-promoted peptidyl transfer reaction

The transfer reactions were performed at 37 °C and analyzed by HPLC and MALDI-TOF/MS. The following RNA oligonucleotides were used as a target (XIAP RNA) and as selectivity control (GAPDH RNA):

XIAP RNA : 5'-GAUAGAUGGCAAUAUGGAGACUCAGCAGUUGGAA -3'

GAPDH RNA : 5'-CCGUCAAGGCUGAGAACGGGAAGCUUGUCAUCAA-3'

10 mM MOPS pH 7.0, 200 mM NaCl2, 0.2 mM TCEP, 0.2 μgmL-1 RiboLock, 37 °C

The experiments were performed by using low binding eppendorf (SafeSeal Microcentrifuge Tubes, *Sorenson BioScience, Inc.*). All manipulations were carried out while minimizing exposure to oxygen. The acceptors (150 μM) were dissolved in a degassed buffer (10 mM MOPS pH 7.0, 200 mM NaCl, 0.2 mM TCEP, 0.2 μgmL-1 RiboLock) which contained 4 mM TCEP. After 30 min, the solution was diluted to the desired final concentration by adding donor PNA conjugate from 110 μM and degassed buffer. The reaction was performed in absence of RNA or in presence of stoichiometric or substoichimetric amounts of RNA. The peptide-PNA conjugates were allowed to react during 3 h. and after this the reaction was quenched with TFA (the final concentration of the TFA was 10%). Considering the stoichiometry of the reaction, the yield was determined by the following expression:

$$\text{Yield} = \frac{\text{Area}_{\text{Product}}}{\text{Area}_{\text{Accepting probe}} + \text{Area}_{\text{Product}}} \times 100$$

Figure S41. Calculation of the yield in the transfer reaction.

Kinetic experiments at stoichiometric template loads

The acceptor (37.5 μM) was dissolved in buffer, which contained 4 mM TCEP. After 30 min 22 μL of this solution was added to 132 μL of the degassed transfer buffer and then 11 μL of a 150 μM solution of donor conjugate was added in the case of absence of RNA. For reactions in presence of RNA, 22 μL of the acceptor solution (37.5 μM) was added to 121 μL of degassed buffer. Subsequently, 11 μL of the donor in buffer (150 μM) and 11 μL of RNA solution (75 μM) were added. Aliquots (15 μL) were withdrawn at different times, quenched by addition of 5 μL of TFA and analyzed by Waters Acquity UPLC *system* (column *Waters-X-Bridge-C18-BEH130* column 1.7μL, 50×2.1mm, pore size 130Å), temperated to 50 °C. Absorbance was measured using a photo diode array detector at 260 nm. Samples were eluted by using solvents A (98.9% water, 1% acetonitrile, 0.1% TFA) and B (98.9% acetonitrile, 1% water, 0.1% TFA) in linear 3% B to 35% B in 2 min gradient at flow rates of 0.6 mL/min. The fitting of the data was carried out using the GraphPadPRISM software.

Kinetic experiments at substoichiometric template loads

In the design of a reaction system that enables turnover under isothermal conditions we considered the distance between the annealing sites on the RNA template and the length of the cognate PNA. Previous experiments revealed that unpaired nucleobases opposite to the ligation site increased the sequence specificity of the PNA ligations[4] and that the maximum transfer rate can be achieved with 3 unpaired nucleotides between the aligned PNA molecules.[5] A suitable pair of reactive PNA conjugates was selected based on measurements of melting temperatures (T_M). In this optimization process the acceptor conjugates were already provided with the cell penetrating peptide. The melting curves obtained for conjugates **29** and **32** showed rather high T_M values (58 °C). We inferred that is would be difficult to enable strand exchange at the 37°C reaction temperature. Therefore, the subsequent experiments were focused on **31**.

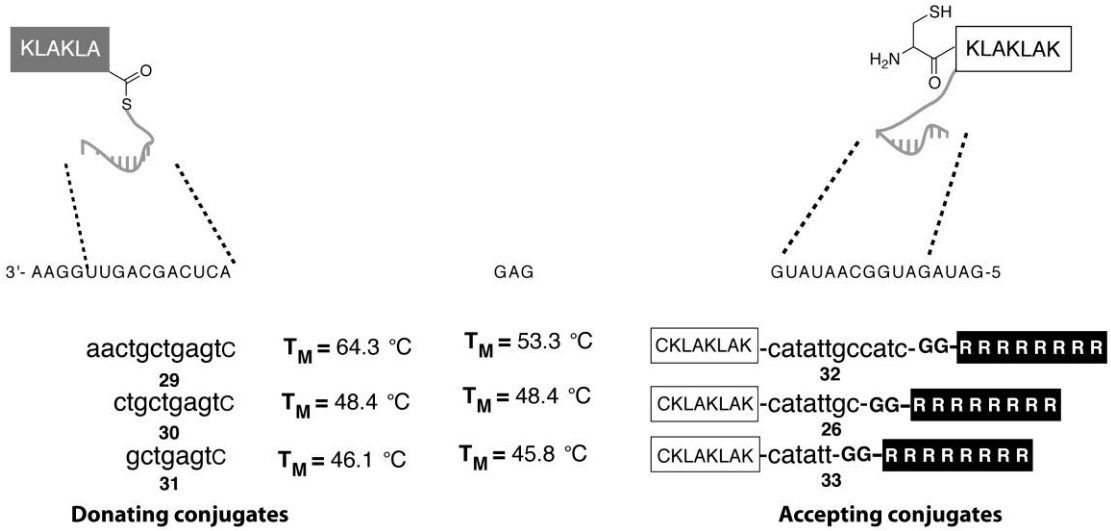


Figure S42. Illustration of the different PNA pairs studied for the optimization of the catalytic transfer reaction.

An acceptor (150 µM **26**, **32** or **33**) was dissolved in buffer, which contained 4 mM TCEP. After 30 min 2 µL of this solution was added to 54 µL of degassed buffer. Then 3 µL of a solution of a donor (200 µM **24**) was added in case the reaction was performed in the absence of RNA. For reactions in presence of RNA, 2.7 µL of a solution of an acceptor (110 µM **26**, **32** or **33**) was added to 52 µL of degassed buffer. Subsequently, 3 µL of the donor solution (**24**, 200 µM) and finally 2 µL of RNA solution (150 µM) were added. The peptide-PNA conjugates were allowed to react for 3 h. The reaction was quenched (6 µL of TFA) and analyzed by *Merck Hitachi* serie Elite LaChrom instrument (column: *Varian Polaris C18* A 5µ 250 × 046, pore size 220 Å). The used eluents were A (98.9% H_2O, 1% acetonitrile, 0.1% TFA) and B (98.9% acetonitrile, 1% H_2O, 0.1% TFA) in the linear 3% B to 50 % B in 30 min gradient with a flow rate of 1 mL/min.

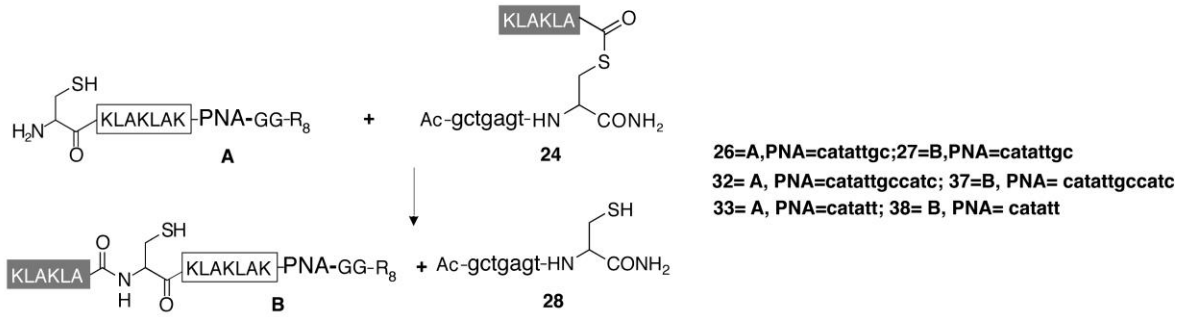


Figure S43. Reactions studied for the optimization of the catalytic transfer reaction.

Figure S44 shows the HPLC analyses of reactions involving the most reactive combinations while the figure S45 shows the HPLC analyses of reactions involving less reactive combinations. The transfer reaction on 0.1 eq template between the shortest length PNA pair (**24**/**33**) provided 60% yield (TON = 5). The yield dropped to 43% (TON = 3) when donor **24** was allowed to react with acceptor **32**.

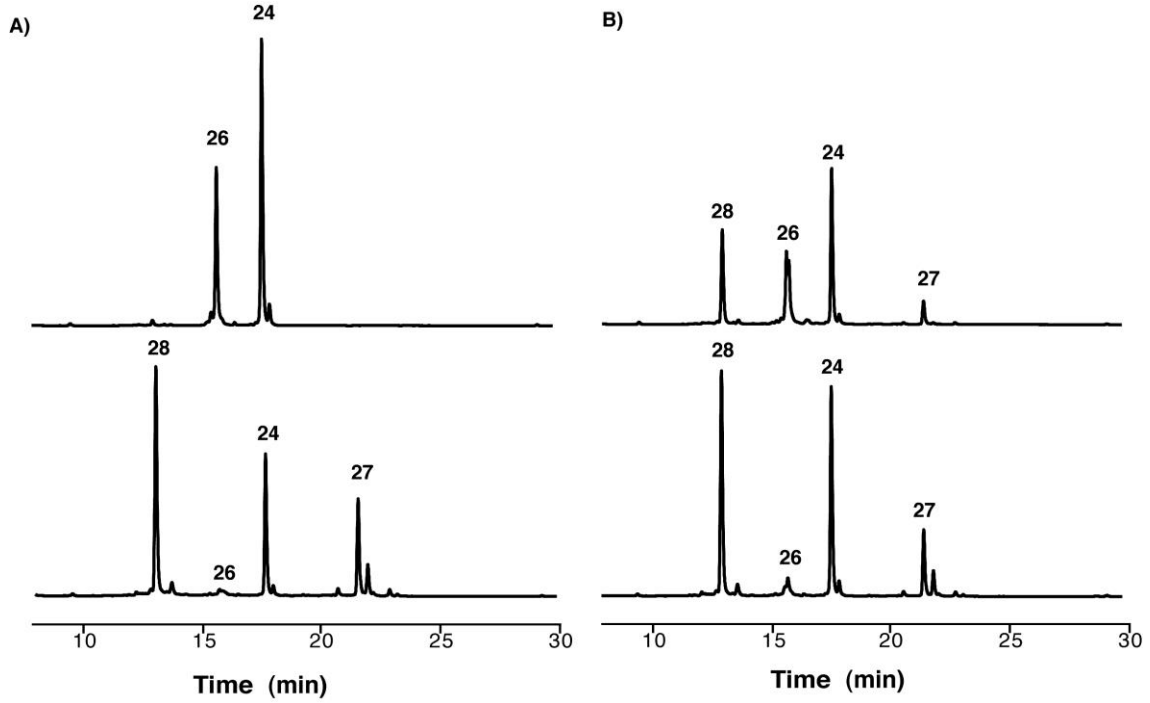


Figure S44. HPLC analysis of the transfer reaction between **24** and **26**. A) Upper figure: reaction at 0 min; lower figure: reaction after 60 min in presence of 1 equiv XIAP RNA. B) Upper figure: reaction after 180 min in absence of XIAP RNA template; lower figure: reaction after 180 min in presence of 0.1 equiv of template. The small peaks next to **27** and **28** were identified as their oxidized derivatives.

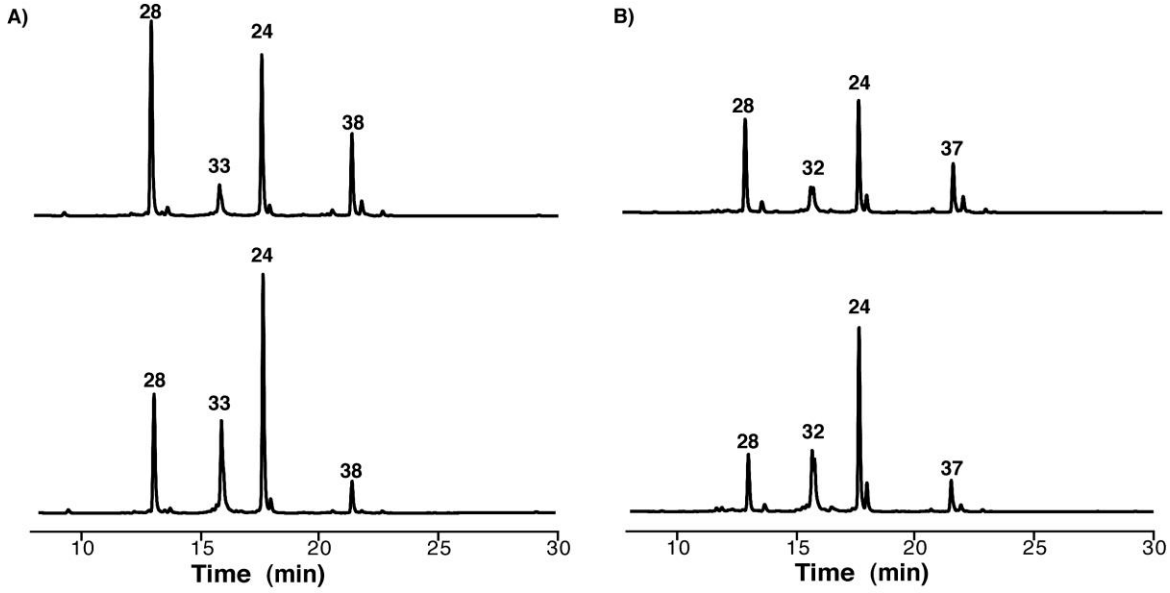
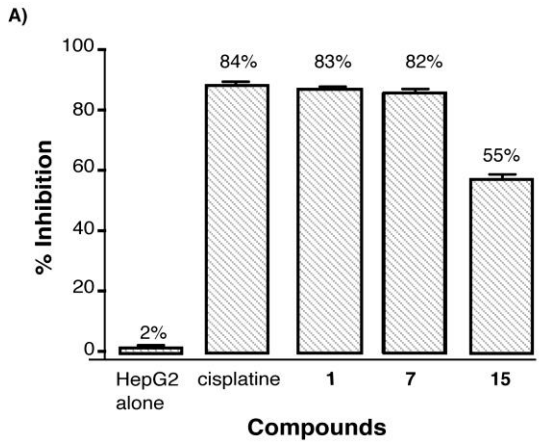


Figure S45. HPLC analyses of reactions involving A) **24** and **33** and B) **24** and **32**. The upper figures show HPLC traces after 180 min reaction in presence of 0.1 eq of XIAP RNA. The lower figures show HPLC traces after 180 min reaction on absence of template

Cell proliferation assay

HepG2 or HeLa cells were grown in DMEM (*Biochrom, Berlin*) supplemented with 1% penicillin streptomycin mixture and 10% FBS (fetal bovine serum) in cell culture flasks at 37 °C with 5% CO_2. The day before assaying cell proliferation, the cells (10000/well) were seeded with DMEM without phenol red (*Biochrom, Berlin*) supplemented with 2.5% FBS in transparent 96-well plates (*TPP®*). Different concentrations of the compounds from 100μM solution in DEM were added to the cells for 16 hours. The cell viability was determined by the CellTiter 96®AQueousOne Solution cell proliferation assay (*Promega*) following the instructions of the manufacturer. Mean values were calculated from three independent experiments.



B)

Compound	% Inh	Compound	% Inh
2	73%	**10**	84%
3	78%	**11**	74%
4	76%	**12**	77%
5	64%	**13**	59%
6	81%	**14**	70%
8	84%	**16**	63%
9	61%	**17**	42%

Figure S46. A) Inhibition of HepG2 cell proliferation by the most promising pair (**7**/**15**) and controls as assessed by the MTS assay. B) Summary table, which lists the inhibition of HepG2 cell proliferation by cysteine mutants of the full- length peptides **2**-**9** and fragments **10**-**17**.

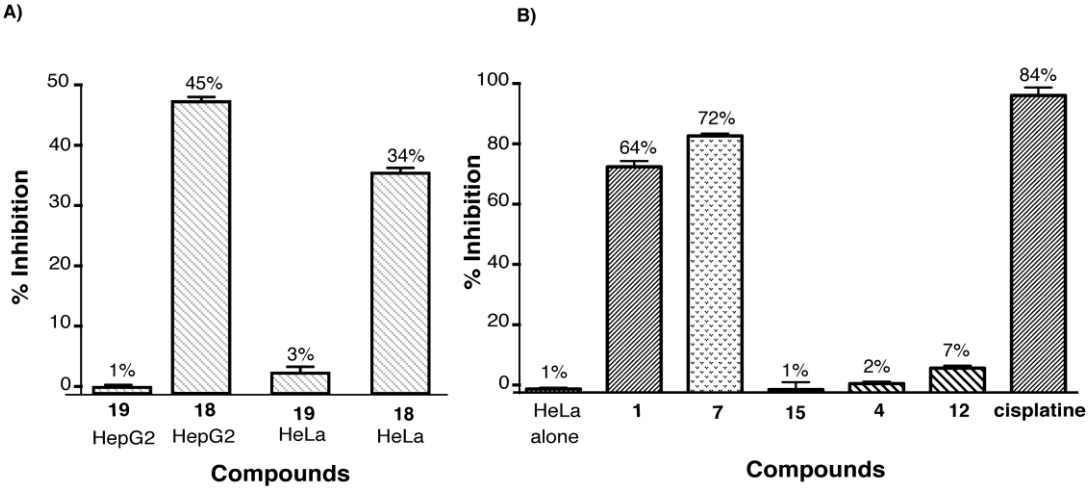


Figure S47. Results of the MTS cell proliferation inhibition assay: A) at 100 μM of the compounds **18** (the peptide without the cell penetrating peptide) and **19** (the octaarginine peptide alone) incubated with HeLa and HepG2 cells; B) at 10 μM of the most promising full length cysteinyl-peptides (**4** and **7**), the short cysteinyl-peptides (**12** and **15**) the controls: **1** and cisplatine) incubated with HeLa.

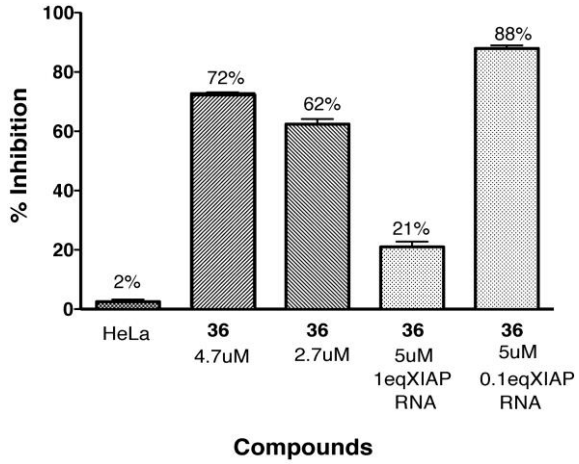


Figure S48. Inhibition of cell proliferation by transfer product **36** in absence and presence of XIAP RNA template.

RNA-programmed peptidyl transfer and coupled cell proliferation assay

Acceptor **26** (2.7 mM) was dissolved in buffer (70 mM MOPS, 200 mM NaCl, 0.2 mg·L^{-1}RiboLock at pH 7.0) containing 4 mM TCEP. After 30 min, 6.4 µL of this solution was added to 476.4 µL of a buffer comprised of 70 mM MOPS, 200 mM NaCl, 0.2 mM TCEP, 0.2 mg·L^{-1} RiboLock at pH 7.0. Subsequently, 17.2 µL of a 2.05 mM solution of donor **24** in buffer (70 mM MOPS, 200 mM NaCl, 0.2 mg·L^{-1}, RiboLock, pH 7.0) was added in case the reaction was performed in absence of RNA. For experiments in presence of RNA, 6.4 µL of the acceptor solution was added to 473.5 µL of buffer before 17.2 µL of the donor solution and 2.9 µL of 608.2 µM solution of RNA (XIAP RNA or GAPDH RNA) was added. The reactions on stoichiometric template were performed by adding 29 µL of 608.2 µM solution of RNA. The peptide-PNA conjugates were allowed to react for 180 min at 37 °C. Afterwards and without any intermediate purification step the reaction was directly added to the cellular media in different concentrations. The reaction, the buffer and all controls were treated in the same way. The cell proliferation assay was performed as explained above.

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