Synthesis of Peptide-PNA-Peptide Conjugates by Semi-Solid-Phase Chemical Ligation Combined with Deactivation/Capture of Excess Reactants

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An expeditious route to peptide-PNA-peptide conjugates following a two-step native chemical ligation (NCL) strategy is described. A cys-PNA-thioester is immobilized on PEGA-aldehyde resin by thiazolidine formation, followed by capping of excess resin aldehydes. The first NCL reaction is then performed with the immobilized PNA-thioester, to give, after release from the solid support, the cys-PNA-peptide intermediate with relatively high purity. The latter is then converted into the target compound by the second NCL reaction with a thioester peptide, the excess of which is captured using a cysteine-PEGA resin. The resulting peptide-PNA-peptide can then be readily isolated by a simple purification step.
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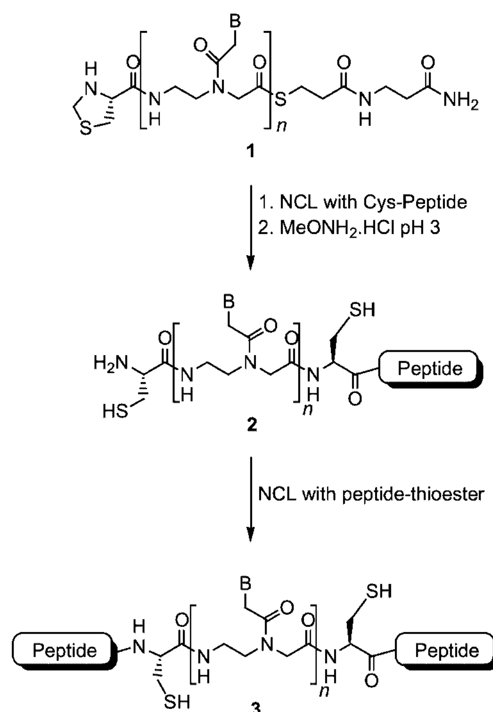
Introduction

Recent advances in biotechnology as well as synthetic peptide and nucleic acid chemistry have opened a way of constructing molecules that may be of great importance as potential therapeutics and biological probes. An example of this progress are peptide nucleic acids (PNAs)[1,2] comprising repeating *N*-(2-aminoethyl)glycine units, the secondary amines of which are attached to purine and pyrimidine nucleobases by a methylenecarbonyl linkage. This non-ionic structural DNA mimic is resistant[3] to nuclease and protease digestion and can hybridize, obeying either Watson−Crick or Hoogsteen base-pairing rules,[2,4−6] to complementary DNA and RNA or dsDNA with higher affinity than their oligonucleotide counterparts. More importantly, PNAs also exert interesting biological effects in the event of binding to DNA or mRNA, including in vitro transcription and translation modulation.[6,7] The aforementioned features make PNAs not only a powerful probe for studying molecular events but also a potential candidate for gene-targeting drugs.[8,9] However, the potential usefulness of PNAs as a therapeutic agent is severely hampered by low cell uptake caused by the poor cell-membrane permeability.[10] Thus far a plethora of invasive approaches to enhance cellular uptake of PNAs have been reported. Physical delivery methods such as microinjection[6] and cell treatment by detergents[11] have been used in studies of antisense or antigene PNAs. Specific delivery of PNAs could also be effected successfully by receptor-mediated cell or tissue internalization.[12−17] On the other hand, PNAs linked to lipophilic groups such as adamantyl,[18,19] phosphonium cation[20] and hydrophobic peptides[21] show significantly improved membrane penetration. It also became apparent that several naturally occurring peptide sequences have the ability to either facilitate cell membrane permeability or nuclear import.[22−24] For example, cell-membrane-permeable



Scheme 1

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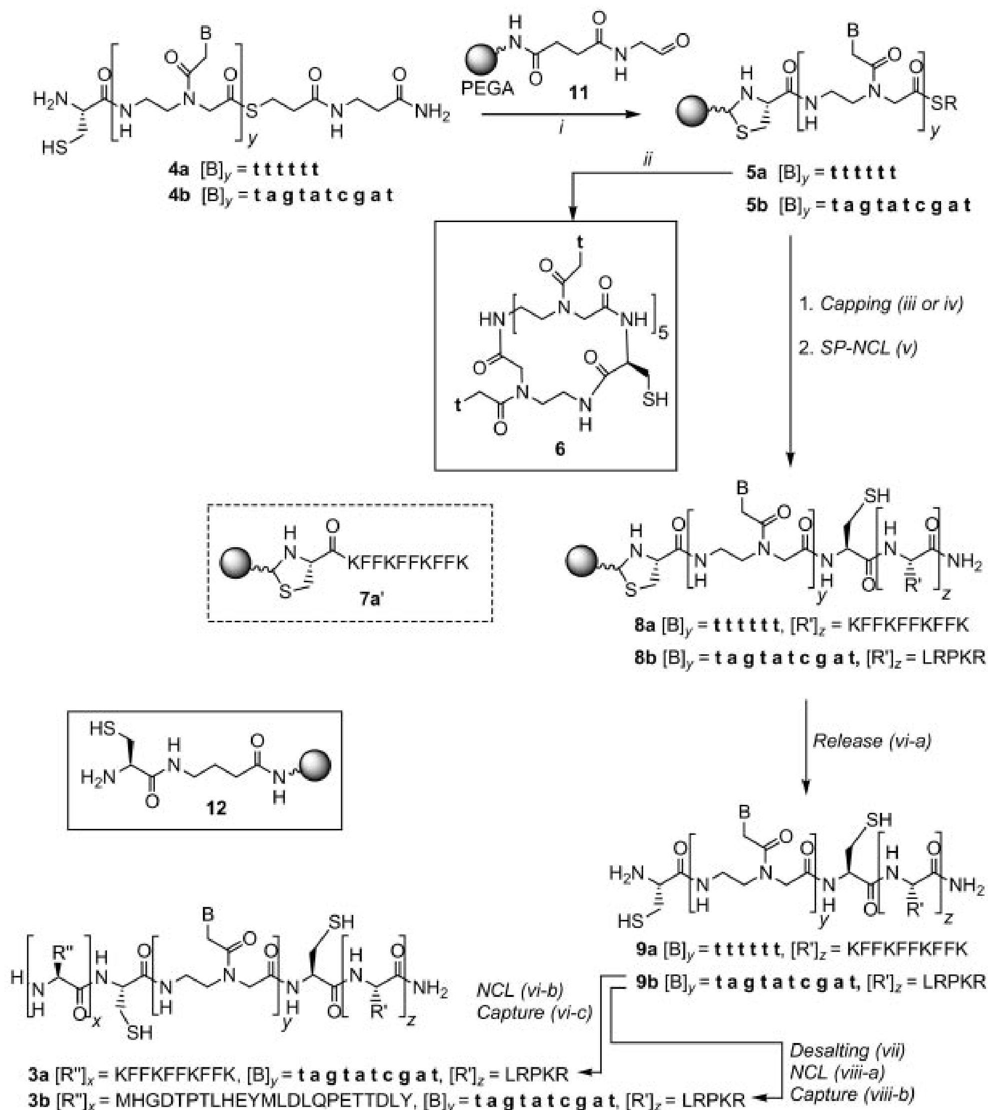
peptides [e.g. pAntp (43−58)][25] linked to either the *C*- or *N*-terminus of PNA enhance cellular uptake. On the other hand, conjugation of PNA to a so-called topogenic sequence [e.g. nuclear localization signal (NLS) sequence of SV40-T][26] facilitates delivery to the nucleus.[27] Thus far, only one example of a PNA construct containing both invasive elements has been reported.[28] In this particular case, the cell-membrane-permeable peptide pAntp was linked by an intracellular cleavable disulfide bond to the nuclear localization signal NLS (SV40-T), which, in turn, was anchored to the *N*-terminus of the target PNA. It was established that internalization and delivery of the PNA is a two-step process. The crucial step proved to be the cleavage of the disulfide bond under the reducing environment in the cytosol, resulting in the exposure[29] of the NLS peptide.

An earlier study[30] from this laboratory showed that the assembly of the PNA **3** (see Scheme 1), carrying two exposed peptide sequences, could, in principle, be realized by a controlled stepwise native chemical ligation[31,32] (NCL) of the bifunctional PNA derivative **1**. Thus, NCL of the *C*-terminal thioester function in **1** with an *N*-terminal Cys-peptide gave, after ring-opening of the *N*-terminal thiaproline unit in the ligation product with *O*-methylhydroxylamine hydrochloride ($MeONH_2$·HCl), and desalting of the reaction mixture, the Cys-PNA-peptide **2**. NCL of the *N*-terminal cysteine unit in **2** with a peptide-α-thioester led to the isolation of **3**. Although the two NCL reactions proceeded in near quantitative yield, isolation of the final ligation product **3** required, due to the use of an excess of both peptides, a time-consuming HPLC purification step, leading



Scheme 2. Reagents and conditions: i) PEGA aldehyde resin **11** (5 equiv.), 0.1 M NaOAc pH = 4.3; ii) a: buffer A, 4 h; b: raise pH from 3 to 7.6, add ca. 10 equiv. of $HSCH_2CH_2COOH$; iii) $BnNH_2$, HOAc, $NaBH(OAc)_3$, DMF, 6 h; iv) 0.15 M $Ph_3P{=}CHCOOEt$ in ACN/H_2O, 1:1, v/v, 15 h; v) **7a** or **7b** (3 equiv.), buffer B pH = 7.6, PhSH (4 vol%), 15 h. **7a**: H-*CKFFKFFKFFK*-NH_2, **7b**: H-*CLRPKR*-NH_2; vi) a: buffer A, 6 h, b: add 3 equiv. of **10a**: H-*KFFKFFKFFK*-C(O)SCH_2CH_2C(O)OEt or raise pH to 7.6, add PhSH (4 vol%), 15 h, c) dilute twice with buffer A, add ca. 10 equiv. of **12** (with respect to thioester), 15 h; vii) desalting using RP-HPLC; viii) a: buffer B, **10b**: H-*MHGDTPTLHEYMLDLQPETTDLY*-C(O)SCH_2CH_2C(O)OEt (3 equiv.), PhSH (4 vol%), 15 h; b: dilute twice with buffer B, add ca. 10 equiv. of **12** (with respect to thioester), 15 h; buffer A: 0.20 M $MeONH_2$·HCl, 6 M guanidine·HCl, 0.1 M TCEP, 0.1 M Na_2HPO_4 pH = 3.1; buffer B: 6 M guanidine·HCl, 0.1 M TCEP, 0.1 M Na_2HPO_4, pH = 7.8

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to unavoidable handling losses. We report here that a semi-solid-phase approach, combined with deactivation/capture of excess reactants, gives access to peptide-PNA-peptide constructs **3** of relatively high purity.

Results and Discussion

A recent study[33] revealed that the cyclic PNA **6** is readily accessible by the solid-phase approach depicted in Scheme 2. In this case, crude cys-PNA-α-thioester **4a** was immobilized at pH = 4.3 by the addition of an excess (5 equiv.) of the water-compatible aldehyde-functionalized polyethylene glycol amine (PEGA) resin[34] **11** to give, after extensive washing, the immobilized thiazolidine-PNA-α-thioester **5a**. Release of **4a** from the solid support under the action of $MeONH_2$·HCl at pH = 3 led, after cyclization at pH = 7.6, to the isolation of high-quality cyclic product **6**. The favorable outcome of this experiment was an incentive to subject **5a**, as a proof of concept, to a solid-phase NCL reaction using crude cys-peptide **7a** for the introduction of the *C*-terminal sequence, which, in turn, facilitates internalization of PNA into *E. coli*.[35,36] Accordingly, a suspension of **5a** in a ligation buffer (0.1 M phosphate buffer pH = 7.6, 6 M guanidine·HCl, 100 mM of reducing agent TCEP) containing the conjugation enhancer thiophenol (4%, v/v) and an excess[37] of peptide **7a** (3 equiv.) was vigorously stirred to the vortex periodically. After 16 h at 20 °C, the ligation product **8a** was isolated by filtration and washed extensively with a mixture of TFA (1%) in acetonitrile/water (1:4, v/v) to remove excess **7a** and other impurities. At this stage, **8a** was suspended in an aqueous solution (pH = 3) of $MeONH_2$·HCl (0.20 M), and shaken gently at 20 °C for 8 h. Analysis of the supernatant by LC/MS showed (see Figure 1A), apart from the expected ligation product **9a**, a small amount of the peptide **7a**. The presence of unwanted[38] **7a** in the supernatant can be explained as follows. The remainder of the excess aldehyde-functionalized PEGA resin used in the immobilization of **4a** also reacts with the cysteine moiety in peptide **7a** to give immobilized **7a′**, which, upon treatment with $MeONH_2$·HCl, leads to the release of **7a**. It was envisaged that this undesirable immobilization could be suppressed by inactivation of excess aldehyde groups on the PEGA resin by reductive amination. To this end, **5a** and residual PEGA-aldehyde resin **11** were subjected to a slight excess of benzylamine and NaHB-$(OAc)_3$ (pH = 6.5) in DMF and vigorously stirred to the vortex periodically at 20 °C for 6 h. The resin was washed thoroughly with DMF and a water/acetonitrile mixture and treated, as mentioned earlier, with NCL followed by release from the support. LC/MS of the supernatant resulting from the treatment of the insoluble materials with $MeONH_2$·HCl showed a drastic decrease in **7a** (see Figure 1B). In addition, the absence of the corresponding benzylamide derivative of **4a** clearly indicates that the thioester function in **5a** is fully compatible with the in situ purification step. It also turned out that Wittig olefination of residual PEGA with [(ethoxycarbonyl)methylene]triphenylphosphorane in acetonitrile/water (pH = 8.5) at 20 °C for 16 h was slightly more efficient than the reductive amination procedure.

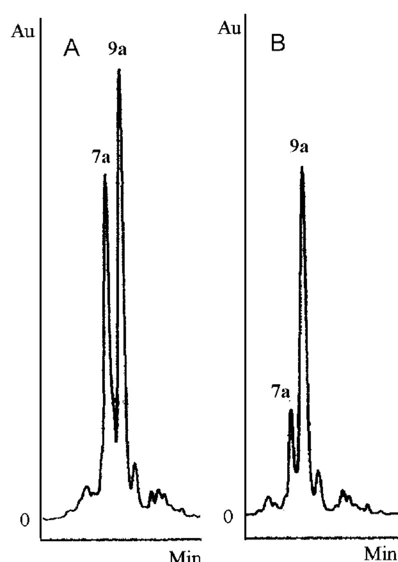

Figure 1. Parts of LCMS traces; A: release of **9a** without capping (control); B: release of **9a** after capping with $NaBH(OAc)_3/BnNH_2$

The aforementioned NCL approach was further demonstrated in the successful synthesis of the PNA-peptide **9b**, which contains a heteromeric PNA sequence linked to the randomly chosen hexapeptide **7b**. Thus, treatment of the bifunctional PNA derivative **4b** with **11**, under the same conditions as for the preparation of **5a**, led to the isolation of immobilized **5b**. Capping of excess PEGA-aldehyde **11** by Wittig olefination and subsequent native chemical ligation of **5b** with excess *N*-terminal cysteine peptide **7b** gave, after processing (i.e. filtration and washing), the immobilized PNA-peptide **8b**. Release of **8b** from the solid support afforded, as gauged by LC/MS (see Figure 2A), crude Cys-PNA-peptide **9b** of high purity. At this stage it is of interest to note that product **9b** is still contaminated with *O*-methylhydroxylamine (HCl salt). It was envisaged that this contaminant, due to its inherently relatively high nucleophilicity, could react in the intended second ligation step with the peptide-α-thioester **10a** to give the corresponding *N*-methoxyamide derivative. Indeed, LC/MS analysis, after 15 h at 20 °C of the ligation reaction of crude **9b** with an excess of crude peptide-α-thioester **10a** under standard conditions (pH = 7.6, thiophenol) revealed, besides complete conversion (see Figure 2B) of **9b** into the target peptide-PNA-peptide conjugate **3a**, the formation of the following three distinct derivatives of **10a**: the *N*-methoxyamide (peak a), the thiophenyl thioester (peak c) and the lactam (peak b). The identity of the lactam and methoxyamide derivatives of **10a** was also indirectly corroborated by capture of the thioesters with the easily accessible immobilized cysteine-PEGA derivative **12**. Thus, treatment of the crude ligation mixture with **12**, at 20 °C for 15 h, led to a near quantitative capture (see Figure 2C) of **10a** and the corresponding thiophenyl ester derivative. Purification of the reaction mixture, resulting after the capture step, by HPLC gave homogeneous

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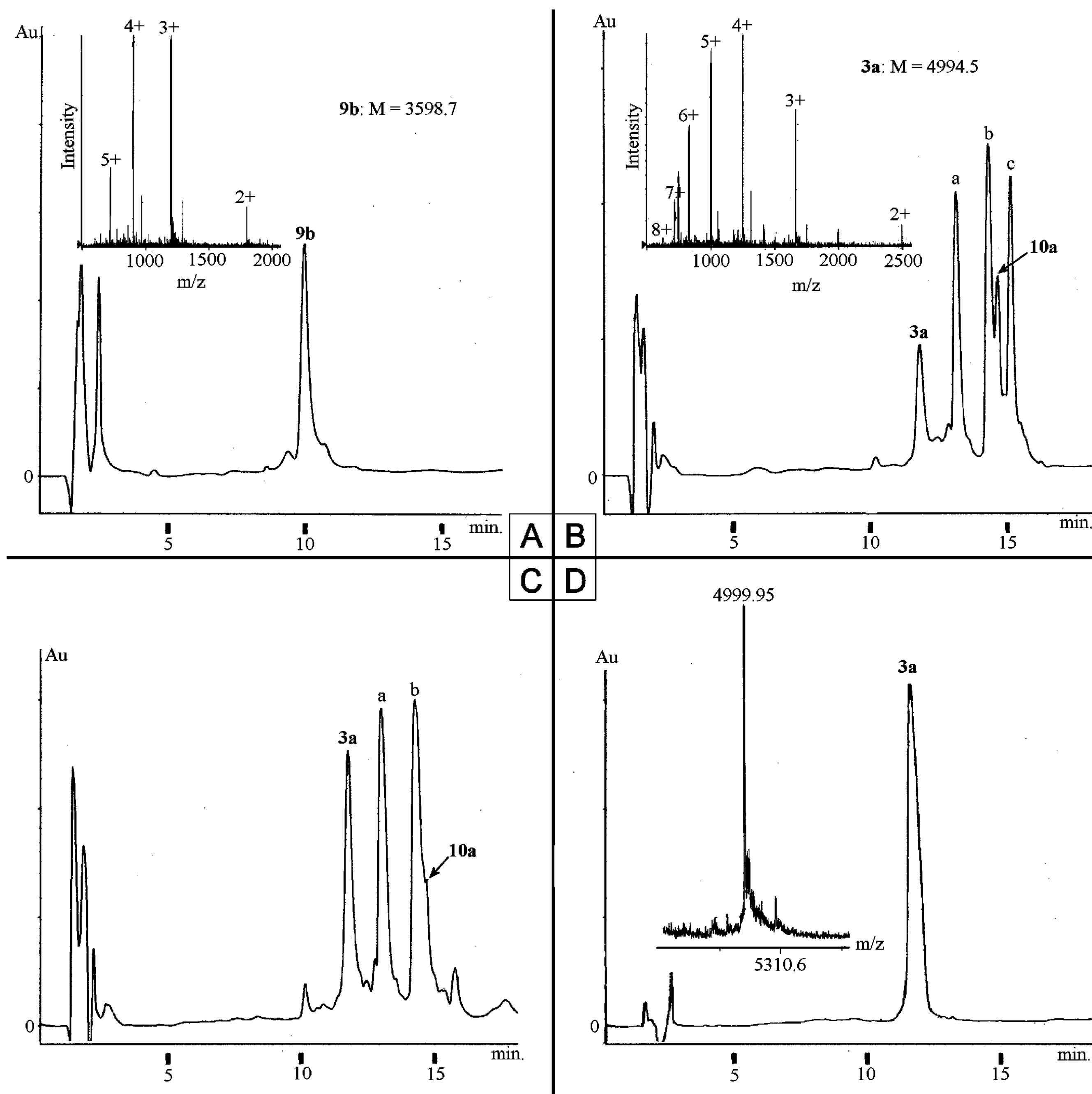

Figure 2. LC traces and MS spectrograms; retention times are in minutes; A: crude **9b** (t_R = 9.9; inset: ESI-MS spectrogram of **9b**); B: ligation mixture after 15 h, containing **3a** (t_R = 11.8; inset: ESI-MS spectrogram of **3a**), peptide-methoxyamide (a, t_R = 13.1), peptide-lactam (b, t_R = 14.3), residual **10a** and peptide-thiophenyl ester (c, t_R = 14.6); C: filtrate after capture of residual peptide thioesters with **12**; D: purified **3a** (inset: MALDI TOF spectrum of **3a**)

target compound **3a**, the identity of which was ascertained by MALDI-TOF spectrometry (see inset in Figure 2D).

In order to evaluate the rate of formation of the unwanted lactam derivative (see Figure 3), peptide **10a** was left under normal NCL conditions (pH = 7.6, excess thiophenol) at 20 °C. Monitoring of the reaction by LC/MS showed a relatively fast conversion (e.g. 20% after 5 h, 50% after 15 h and 80% after 48 h) of **10a** into the corresponding lactam derivative. The ease of formation of the lactam is mainly due to intramolecular attack of the ε-NH_2 function of the *C*-terminal lysine unit in the activated thiophenyl thioester of **10a** (see pathway a in Figure 3). Alternatively, intramolecular attack (pathway b) of the ε-NH_2 on the thioester function in **13**, resulting from the chemoselective step between **10a** (R = Ph) with the Cys-PNA-peptide **9b**, is kinetically disfavored over the α-NH_2 attack (pathway c) leading to the expected ligation product **3a**. Additional evidence in support of the structure of the lactam side-product was obtained from two-dimensional NMR experiments (TOCSY, COSY), which showed that the expected triplet

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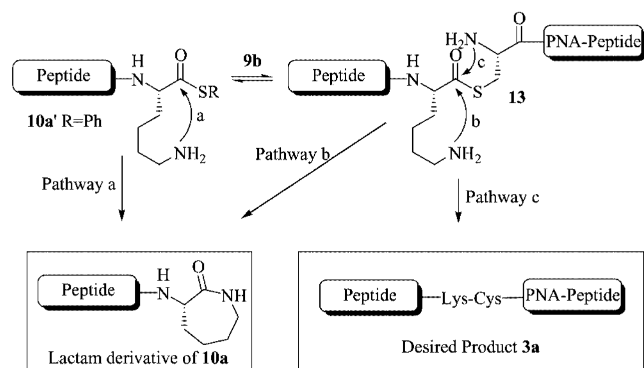

Figure 3. Possible pathways for the formation of the **10a**-derived lactam side product

(δ = 7.9 ppm) of the lactam amide proton couples with the ε-CH_2 group of the *C*-terminal lysine residue.

Although the capture of residual peptide-α-thioesters with **12** had a beneficial effect on the final purification, it is not excluded that the presence of the *N*-methoxyamide derivative may hamper a straightforward purification. However, the latter obstacle can be nullified by the removal of excess *O*-methylhydroxylamine (HCl salt) by a simple desalting procedure. For example, NCL of desalted **9b** with an excess of peptide **10b**, containing a *C*-terminal tyrosine-α-thioester, at 20 °C for 15 h, was followed by the addition of cys-PEGA **12**, and left at 20 °C for 15 h. LC/MS analysis of the sample obtained after removal of the insoluble materials by filtration led to high-quality **3b** (see Figure 4A). Purification by RP-HPLC afforded homogeneous peptide-PNA-peptide **3b**, the structure of which was corroborated by ESI-MS and MALDI-TOF spectrometry (see insets B and C in Figure 4).

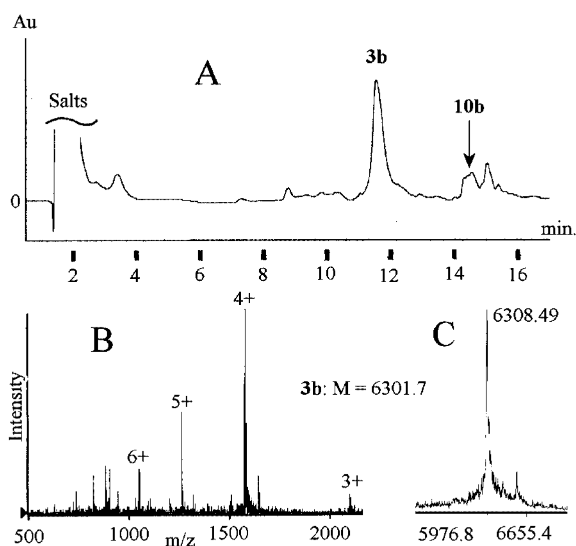

Figure 4. A: LC/MS trace of crude **3b** (after capture of residual thioesters), t_R = 11.5 min; B: ESI-MS spectrogram of crude **3b**; C: MALDI TOF spectrogram of purified **3b**

Conclusion

The results in this paper show that time-consuming and labor-intensive purification steps, after a stepwise NCL of a Cys-PNA-thioester (**4**) with an *N*-terminal cysteine peptide and a *C*-terminal thioester peptide can, in principle, be prevented by a synthesis protocol involving a solid- and a solution-phase native ligation in combination with the removal of excess reagents by capture, capping and an optional desalting step. It is also evident that this protocol not only allows the use of crude Cys-PNA-thioesters but also an excess of *N*-terminal cys-peptides as well as *C*-terminal thioester peptides. The successful construction of peptide-PNA-peptides may also open the way for application of the same methodology for the synthesis of polypeptides starting from cys-peptide-α-thioesters. In this respect it is noteworthy that the use of the agarose-based aldehyde resin AminoLink® (Pierce) would be more advantageous, because of its full compatibility with thioester ligation[39] and high MW peptides, than the PEGA-aldehyde resin.

We also established that the lysine thioester peptide **10a** rearranges under common ligation conditions into the corresponding lactam derivative (e.g. 20% lactamization occurred within 5 h). Since this result is unprecedented for commonly used lysine thioesters[40−43] it is not excluded that the lactamization of peptide **10a** may be dependent on the peptide composition.

At present we are studying the antisense/antigene properties of peptide-PNA-peptides as well as the scope of our novel methodology. Both aspects will be reported in due time.

Experimental Section

General Remarks: Abbreviations: ACN: acetonitrile; Boc: *tert*-butoxycarbonyl; BOP: benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate; DCM: dichloromethane; DiPEA: *N*,*N*-diisopropylethylamine; DMF: *N*,*N*-dimethylformamide; DMSO: dimethyl sulfoxide; Fmoc: 9-fluorenylmethoxycarbonyl; HATU: *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HOBT: *N*-hydroxybenzotriazole; LC/MS: liquid chromatography/mass spectrometry. MALDI TOF: matrix assisted laser desorption ionisation time of flight; MBHA: methylbenzhydrylamine; NCL: native chemical ligation; NMP: *N*-methylpyrrolidone; Pbf: 2,2,4,6,7-pentamethyldihydrobenzofuran-5-ylsulfonyl; PEGA: polyethylene glycolamine; PNA: peptide nucleic acid; PS: polystyrene; RP-HPLC: reversed phase high performance liquid chromatography; SPPS: solid phase peptide synthesis; TCEP: tris(2-carboxyethyl)phosphane; TFA: trifluoroacetic acid; THF: tetrahydrofuran; TIS: triisopropylsilane; TFMSA: trifluoromethanesulfonic acid; Trt: trityl (triphenylmethyl); Z: benzyloxycarbonyl. All reagents were of analytical or peptide synthesis grade and used as obtained from the suppliers. All solvents were of HPLC grade (ACN) or peptide-synthesis grade. Analytical LC/MS was conducted with a JASCO system using an Alltima C_{18} analytical column (5 μm particle size; flow: 1.0 mL/min). The absorbance was measured at 214 and 254 nm. Solvent system: A: 100% water, B: 100% ACN, C: 0.5% TFA. Gradients of B in A/C (9:1) were applied over 15 min. Mass spectra were recorded with a Perkin−Elmer

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Sciex API 165 equipped with an electrospray interface (ESI). MALDI-TOF-MS spectra were recorded with a Voyager-DE PRO mass spectrometer (PerSeptive Biosystems) using a α-cyano-4-hydroxycinnamic acid matrix (10 mg/ml 0.2% TFA in ACN/water, 1:1). Purifications were conducted using a BioCAD "Vision" automated HPLC system (PerSeptive Biosystems, Inc.), supplied with a semi-preparative Alltima C_{18} column (5 μ particle size, running at 4.7 mL/min). Gradients of acetonitrile (B) in A/C (9:1) were applied over three column volumes (CV). Solvent system: A: 100% water, B: 100% ACN, C: 1% TFA. A TitroLine alpha machine or Merck Universalindikator pH 1−10 paper was used to measure the pH of buffers. The peptide and PNA fragments were prepared using an ABI 433A (Applied Biosystems) automatic peptide synthesizer. ^{1}H NMR spectra were recorded with a Bruker DMX-600 spectrometer at 273 K. Chemical shifts (δ) are relative to the solvent peak of CD_3OH (δ = 3.30 ppm). Notation for masses: $[M + x H]^{x+}$: found mass (calcd. av. mass).

Synthesis of cys-Peptides 7a,7b: Peptides were synthesized on a 50-μmol scale on Tentagel® Rink amide resin according to standard SPPS protocols using the Fmoc strategy. BOP/HOBT/DiPEA was used as the coupling reagent for the amino acids [Fmoc-Lys(Boc)-OH, Fmoc-Phe-OH, Boc-Cys(Trt)-OH, Fmoc-Leu-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Pro-OH]. For the last coupling Boc-Cys(Trt)-OH was used. After cleavage from the resin, LC/MS indicated that the peptides were of sufficient purity for further processing.

Synthesis of Peptide-Thioesters 10a, 10b: Peptide-thioesters were synthesized according to the procedures described by Pessi et al.[44] Thus, peptide elongation (BOP/HOBT/DiPEA) was carried out on a 50-μmol scale on Kenner's safety-catch resin using *N*-Fmoc-protected amino acids having the following side-chain protections: Lys(Boc), His(Trt), Asp(*t*Bu), Thr(*t*Bu), Glu(*t*Bu), Tyr(*t*Bu) and Gln(Trt). For the final coupling an *N*-Boc-protected amino acid was used. Alkylation of the sulfonamide resin was accomplished by swelling the resin in THF and subsequent treatment with 1 M trimethylsilyldiazomethane in THF/hexane (1:1) for 2 h. After several washing steps (THF, DMF), cleavage from the resin was effected by shaking a suspension of the alkylated resin in 1 M ethyl 3-mercaptopropionate in DMF in the presence of a catalytic amount of sodium thiophenolate for 36 h. After filtration and concentration, the oily residue was treated with TFA/TIS/water (95:2.5:2.5, 5 mL) for 2 h. The peptide-thioester was precipitated in cold diethyl ether and centrifuged. After a second washing step with diethyl ether and centrifugation, the pellet was dissolved in water and lyophilized. The crude peptide thioester **10a** proved to be of sufficient quality for further processing. LC/MS: 5−55% B, t_R = 14.51 min. ESI-MS: m/z = $[M + H]^{+}$: 1531.0 (1529.8); $[M + 2 H]^{+}$: 766.1 (765.4); $[M + 3 H]^{+}$: 511.1 (510.6). The 23-mer peptide-thioester **10b** was purified by RP-HPLC (10−50% B). LC/MS: 10−55% B, t_R = 14.32 min. ESI-MS: m/z = $[M + H]^{+}$: 2836.2 (2837.2); $[M + 2 H]^{2+}$: 1419.6 (1419.6).

Synthesis of cys-PNA-Thioesters 4a, 4b: Commercially available methylbenzhydrylamine resin (0.62 mmol/g; 2 g, 1.24 mmol) was swollen in DMF (20 mL) for 15 min. Fmoc-βAla-OH (0.5 equiv., 193 mg), BOP (0.5 equiv., 274 mg) and DiPEA (3 equiv., 630μL) were added and the mixture was shaken for 2 h. The resin was filtered and washed with DMF and DCM and air-dried. The loading of the resin (0.25 mmol/g) was determined by standard Fmoc quantification. The resin was swollen in DMF and treated with a solution of acetic anhydride (0.5 M) and DiPEA (0.125 M) in DMF (20 mL, 5 min). The resin was washed with DMF and DCM and dried. 1 g (0.25 mmol) of this resin was swollen in DMF (10 mL), drained and treated with 15 mL of 20% piperidine in DMF (2 × 10 min) After washing of the resin with DMF, *S*-tritylpropionic acid (5 equiv., 435 mg, prepared as described)[45] was preactivated for 2 min with HATU (4.6 equiv., 437 mg) and DiPEA (10 equiv., 425 μL) in DMF (5 mL) and added to the resin and the mixture was shaken for 1 h. After washing the resin with DMF, a negative Kaiser test indicated complete consumption of the amine groups. Any unchanged amines were capped with 0.5 M acetic anhydride and 0.125 M DiPEA in DMF (5 min). After washing with DMF and DCM the resin was dried in an air flow. The resin (0.5 g, 0.125 mmol) was swollen in DCM and treated with a solution of TFA/TIS/DCM (5:5:90, 4 mL) until the initial color had disappeared. The resin was filtered, washed with DCM and the deprotection step was repeated for another 10 min. After filtration and washing with DCM, the Ellman test on a small aliquot of the resin indicated the presence of free thiol groups. The resin was washed and swollen in DMF and the solvent was drained. Then, a solution in DMF (5 mL) was prepared containing Boc-PNA thymine monomer (Boc-t-OH, 5 equiv., 260 mg), HATU (4.98 equiv., 255 mg) and DiPEA (10 equiv., 230 μL), which, after 1 min of pre-activation, was added to the resin and shaken for 1 h. The resin was filtered, washed (DMF, DCM), and the Ellman test was negative. The resin was capped using 0.125 M DiPEA and 0.5 M Ac_2O in DMF (1 min) and after washing with DMF and DCM the resin was dried by air. PNA synthesis was carried out on a 10-μmol scale (50 mg of the dried resin) either manually or fully automated using an ABI433a peptide synthesizer. Boc deprotection was effected by a 15-min treatment with 50% TFA/DCM. After thorough washing with DCM and DMF, the Boc/Z-protected PNA monomers (5 equiv.) were pre-activated for 1 min with HATU (4.9 equiv.) and DiPEA (10 equiv.) in DMF (0.8 mL) and added to the resin. The resin was shaken for 30 min, drained and washed with DMF, followed by a 1-min capping step (cap solution: Ac_2O/2,6-lutidine/NMP, 5:6:89). The final coupling with Boc-Cys(Trt)-OH was carried out using the same conditions. After complete synthesis, the resin was washed with DCM, dried, transferred into a glass tube and suspended in TFA (3.2 mL) and TIS (400 μL). The resulting mixture was cooled in an ice-bath followed by dropwise addition of TFMSA (400 μL) under a stream of argon. The mixture was kept at 0 °C for additional 5 min and was then allowed to warm to room temperature and shaken for 1.5−2 h. The suspension was filtered into cold diethyl ether and the resin was washed with neat TFA. The resulting suspension was centrifuged and the diethyl ether layer was decanted. The precipitate was washed with diethyl ether and after centrifugation, the diethyl ether was removed and the precipitate was dissolved in water, lyophilized and used without further purification.

4a: Crude yield: 12 mg. LC/MS: 5−30% B, t_R = 15.4 min. ESI-MS: m/z = $[M + H]^{+}$: 1878.4 (1877.9); $[M + 2 H]^{2+}$: 939.4 (940.0).

4b: Crude yield: 18 mg. LC/MS: 5−40% B, t_R = 10.63 min. MALDI TOF MS: m/z = $[M + H]^{+}$: 3004.76 (3004.99). ESI-MS: m/z = $[M + 2 H]^{2+}$: 1503.6 (1503.0); $[M + 3 H]^{3+}$: 1002.4 (1002.3); $[M + 4 H]^{4+}$: 752.0 (752.0).

General Procedure for the Capture of cys-PNA-Thioesters 5a, 5b: PEGA-aldehyde resin, prepared as described previously,[34] was filtered from the stock suspension and washed subsequently with water, EtOAc and DCM. After drying by an air flow, 5 equiv. (0.4 mmol/g) of resin were added to a solution (5 mM) of crude cys-PNA-thioester in NaOAc buffer (0.1 M, pH = 4.3) containing TCEP (50 mM). The suspension was shaken for 15 h and filtered. The residue was washed thoroughly with 1% TFA in water/ACN (1:1) to remove any contaminants. The resin (**5a**, **5b**) was stored at 4 °C.

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Capping. Reductive Amination Procedure: Resin **5a** (5.7 mg, ≤ 2.3 μmol aldehyde groups) was swollen in DMF. Next, a solution (0.5 mL) of benzylamine (40 equiv., 10μL), HOAc (80 equiv., 105 μL) and $NaHB(OAc)_3$ (40 equiv., 20 mg) was added to the resin and shaken gently at 20 °C for 6 h. The solution was drained and the resin was washed subsequently with DMF and water. **Wittig Olefination:** Resin **5b** (50 mg, ≤ 20 μmol aldehyde groups) was swollen in ACN/water (1:1) and treated with a solution of [(ethoxycarbonyl)methylene]triphenylphosphorane (0.15 M, 700 μL) at 20 °C for 16 h. The resin was washed with ACN/water (1:1) and stored at 4 °C.

General Procedure for the Solid-Phase NCL (8a/8b): The resin (**5a**, **5b**) was swollen in ligation buffer (0.1 M Na_2HPO_4 pH = 7.8, 0.1 M TCEP, 6 M guanidine·HCl). In the meantime, a 1−3 mM solution of the cys-peptide (**7a/7b**, 3 equiv. of peptide with respect to the PNA) in the ligation buffer was prepared (pH drops to 7.6). After filtration of the swollen resin, the peptide solution was added to the resin followed by the addition of PhSH (4%). The vial was sealed and shaken at 20 °C for 16 h. After filtration and intense washing of the resin with water, ACN and finally 1% TFA in water/ACN (1:1), the resin was stored at 4 °C.

Preparation of Resin 12: Wet (MeOH) PEGA resin (2.5 g, 0.125 mmol) was washed with DMF. *N*-Fmoc-4-aminobutyric acid (203 mg, 0.625 mmol) was dissolved in DMF (4 mL), HATU (226 mg, 0. 594 mmol) and DiPEA (213 μL, 1.25 mmol) were added and the mixture was left to preactivate for 1 min. The solution was added to the resin and allowed to react for 1 h. The mixture was drained and the resin was washed several times with DMF. A quantitative Fmoc determination indicated an Fmoc substitution of 0.4 mmol/g of dry resin. Next, the resin was treated twice with 20% piperidine in DMF (5 mL) for 5 min and the resin was washed again with DMF. A solution of Boc-Cys(Trt)-OH (290 mg, 0.625 mmol), HATU (226 mg, 0.95 mmol) and DiPEA (213 μL, 1.25 mmol) in DMF (4 mL) was preactivated for 1 min and added to the resin. The resulting mixture was shaken for 1 h, drained and the resin was washed subsequently with DMF and DCM. A quantitative conversion of free amines was assumed since the Kaiser test on a few beads of resin was negative. The resin was treated with 50% TFA in DCM (5 mL) containing TIS (10%) for 20 min. After draining the solution, the resin was washed subsequently with DCM and water and stored as a slurry in water/ACN (1:1) at 4 °C.

Peptide-PNA-Peptide 3a: The resin **8b** (25 mg) was washed with water and allowed to react in a buffer (400 μL) at pH = 3.1, containing $MeONH_2$·HCl (0.2 M), Na_2HPO_4 (0.1 M), TCEP (0.1 M) and guanidine·HCl (6 M), at 20 °C for 8 h. The resin was removed by filtration and the pH of the filtrate was raised to 7.9 by careful addition of 2 N NaOH. Peptide-thioester **10a** (4.5 mg, 3 μmol) and thiophenol (4%) were subsequently added, resulting in a decrease of the pH to 7.6, and the mixture was shaken at room temperature for 15 h. Next, cysteine-resin **12** (75 mg, ca. 10 equiv. with respect to **10a**) and extra ligation buffer (400 μL) were added to the reaction mixture and left for 15 h. The resin was removed by filtration and washed with ACN/water (1:4, total of 1.5 mL). The combined filtrates were applied to HPLC (10−55% B) which yielded, after concentration and lyophilization, pure **3a** (0.9 mg) as a white, fluffy solid.

Intermediate 9b: LC/MS: 5−50%, t_R = 9.93 min. MALDI TOF MS: *m/z* = $[M + H]^+$: 3599.0 (3599.7). ESI-MS: *m/z* = $[M + 2H]^{2+}$: 1800.4 (1800.4); $[M + 3 H]^{3+}$: 1200.6 (1200.6); $[M + 4 H]^{4+}$: 901.0 (900.7); $[M + 5 H]^{5+}$: 720.8 (720.7).

3a: LC/MS: 10−55% B, t_R = 11.71 min. MALDI TOF MS: *m/z* = $[M + H]^+$: 4999.95 (4994.50). ESI-MS: *m/z* = $[M + 2 H]^{2+}$: 2499.8 (2498.2); $[M + 3 H]^{3+}$: 1666.2 (1665.8); $[M + 4 H]^{4+}$: 1249.8 (1249.6); $[M + 5 H]^{5+}$: 999.8 (999.9); $[M + 6 H]^{6+}$: 833.8 (833.4); $[M + 7 H]^{7+}$: 714.8 (714.5); $[M + 8 H]^{8+}$: 625.6 (625.3).

Peptide-PNA-Peptide 3b: Resin **8b** (25 mg) was treated with the release buffer as described above. After filtration and washing of the resin with water (ca. 1 mL in total), the combined filtrates were desalted by HPLC (5−40% B). After concentration of the fractions containing the PNA material (**9b**), the residue was dissolved in ligation buffer [Na_2HPO_4 (0.1 M), TCEP (0.1 M) and guanidine·HCl (6M), pH = 7.8, 500 μL]. Peptide-thioester **10b** (8.5 mg, 3 μmol) was added as well as thiophenol (4%) and the mixture was shaken at room temperature for 15 h. Excess thioesters were captured as described for the preparation of **3a**. HPLC purification (10−55% B) yielded, after lyophilization, pure **3b** (0.8 mg) as a white solid.

LC/MS: 10−55% B, t_R = 11.33 min. MALDI TOF MS: *m/z* = $[M + H]^+$: 6308.5 (6301.7). ESI-MS: *m/z* = $[M + 3 H]^{3+}$: 2102.2 (2101.6); $[M + 4 H]^{4+}$: 1576.8 (1576.7); $[M + 5 H]^{5+}$: 1262.0 (1261.5); $[M + 6 H]^{6+}$: 1051.4 (1051.5); $[M + 7 H]^{7+}$: 901.4 (901.4).

Lactam Derivative of 10a: Peptide-thioester **10a** (3 mg, 2 μmol) was dissolved in ligation buffer [Na_2HPO_4 (0.1 M), TCEP (0.1 M) and guanidine·HCl (6 M), pH = 7.8, 500 μL]. Thiophenol (20 μL, 4%) was added and the mixture was shaken for 48 h. Aliquots (10 μL) of the reaction mixture were taken after 5, 15 and 48 h, diluted with 1% TFA to 70 μL and analysed by LC/MS (10−55%). The lactam was purified by semi-preparative HPLC (15−55% B) to yield a fluffy white solid (1 mg, 0.7 μmol). LC/MS: 15−50% t_R = 14.31 min. ESI-MS: *m/z* = $[M + H]^+$: 1397.2 (1396.7); $[M + 2 H]^{2+}$: 698.9 (698.9); $[M + 3 H]^{3+}$: 466.1 (466.2). See the Supporting Information for ^1H, TOCSY and COSY spectra of this compound.

Supporting Information: ^1H and parts of the TOCSY and COSY NMR spectra of the lactam derivative of peptide **10a**, see footnote on the first page of this article.

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