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An approach to the synthesis of peptide–PNA–peptide conjugates via native ligation

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Abstract—A convenient solid-phase synthesis of a PNA sequence containing an N-terminal thiaproline and a C-terminal thioester is described. The usefulness of this bifunctional PNA molecule is illustrated by the construction, based on native ligation, of a peptide–PNA–peptide adduct. © 2002 Elsevier Science Ltd. All rights reserved.

Peptide nucleic acids (PNAs), which are non-ionic DNA mimics,[1–3] are resistant to nuclease and protease digestion and hybridize to complementary DNA/RNA sequences with higher affinity than DNA oligomers. The latter features have led to the successful application of PNA as antisense agents in vitro.[1,4] Unfortunately, the inability of PNA to cross cellular membranes severely hampers the evaluation of their therapeutic potential in vivo. It has been established, that cell membrane permeable peptides covalently linked to either the C- or N-terminus of PNA enhance cellular uptake.[5] In addition, conjugation of PNA to a so-called topogenic peptide sequence facilitates the delivery to the nucleus.[6] Recently, Braun et al.[7] prepared a peptide–PNA conjugate (see Fig. 1) in which the PNA unit is linked at the N-terminus to a nuclear localization signal (NLS) peptide, which in turn is anchored via an intracellular cleavable disulfide bond to the membrane permeable peptide pAntp.[8] It was established, that this modular shuttle was not only translocated into tumor cells but also into the nucleus after intracellular disruption of the disulfide bond under the reducing environment in the cytosol. Interestingly, a structurally related hybrid containing a non-degradable bond between pAntp and the NLS peptide failed to cross the nuclear membrane barrier, indicating that the presence of the NLS peptide in an accessible form is essential.

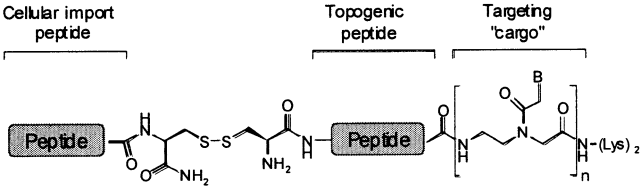

Figure 1.

It occurred to us that a PNA construct containing both exposed peptides as in **1** (see Scheme 1) would obviate the requirement of liberating the NLS peptide by intracellular cleavage of the disulfide bond. A recent contribution from our laboratory[9] revealed that a peptide thioester could be regioselectively ligated to a PNA carrying an N-terminal cysteine. Moreover, it was reported[10a] that an N-terminal thiaproline can be readily converted into cysteine using *O*-methyl hydroxylamine hydrochloride. A retrosynthetic analysis of **1**, based on the above mentioned information, indicates that the bifunctional PNA derivative **4** carrying a C-terminal thioester and an N-terminal thiaproline, would be ideally suited for the construction of **1**. Thus, ligation of **4** with the N-terminal cysteine peptide **5**, and ensuing transformation of the N-terminal thiaproline into the required cysteine would give Cys–PNA–peptide **3**. Ligation of **3** with peptide thioester **2** would then lead to the trimeric target compound **1**. We here wish to report a convenient solid phase synthesis of the bifunctional PNA derivative **4** and its use in a solution phase preparation of **1**.

The assembly of the bifunctional heptameric PNA unit **4** is depicted in Scheme 2 and commences with the preparation of the immobilized *S*-trityl protected linker **6**. In this respect it is of interest to note that the incorporation of extra spacer length between the sulfur

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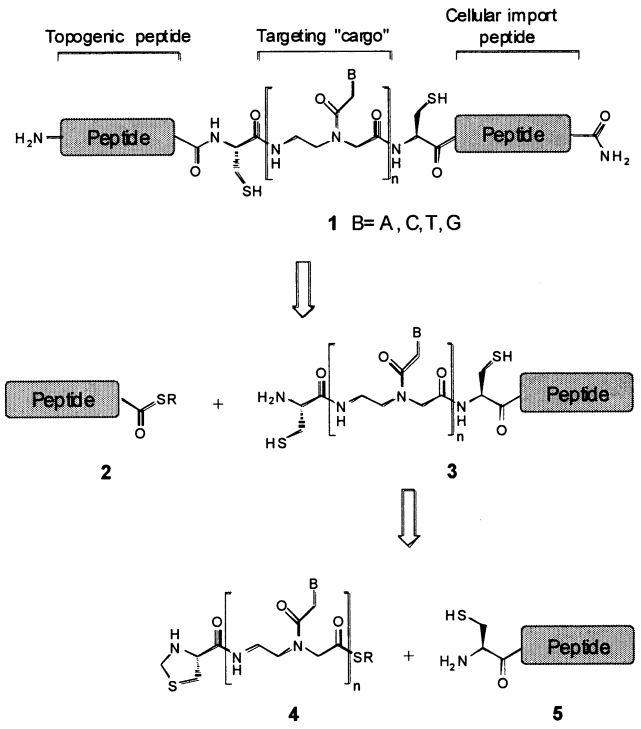
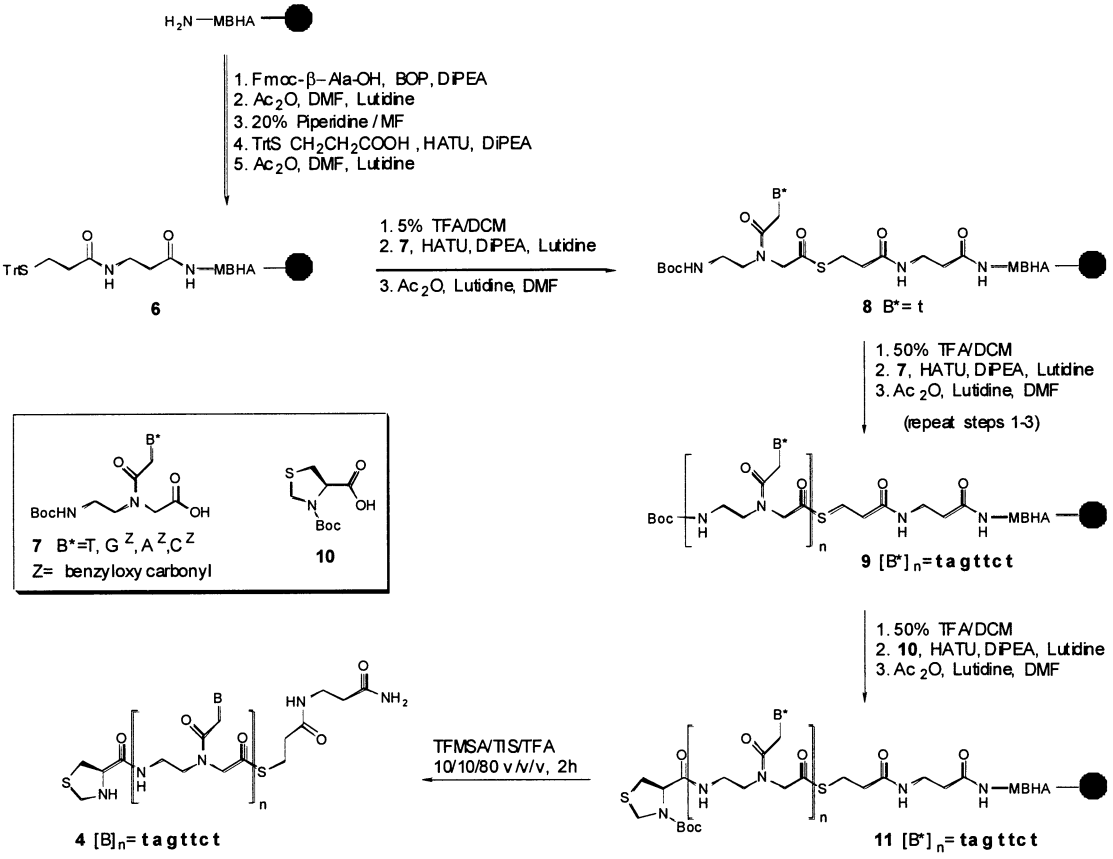


Scheme 1.

atom and the 4-methylbenzhydrylamine (MBHA) resin enhances the stability of the linker.[11] To this end, commercially available MBHA resin was loaded with 9-fluorenylmethyloxycarbonyl(Fmoc)-β-alanine by the action of Castro's reagent[12] (BOP) in the presence of diisopropylamine (DiPEA). Unmasking of the Fmoc group with 20% piperidine and condensation of the free amino group with known *S*-trityl-propionic acid[13] under the agency of the more powerful peptide coupling reagent *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU)[14] afforded immobilized **6** having a loading capacity of 0.25 mmol/g. Detritylation of **6** under mild acidic conditions (5% TFA in DCM) was followed by HATU-mediated coupling of the free thiol group with the commercially available *N*-*tert*-butyloxycarbonyl(Boc)-protected thymidinyl PNA monomer **7** (B* = t) to give the immobilized PNA thioester derivative **8**. Although subjection of **8** to the Ellman test showed the absence of thiol groups, possible residual thiols were capped with acetic anhydride. Sequential elongation of **8** with the respective PNA monomers **7** to give the heptameric PNA thioester **9** was carried out by repeating (six times) the following fully automated three-step synthesis protocol. Thus, acidolysis of the N-terminal Boc protecting group in **8** was followed by HATU-assisted condensation of the respective incoming PNA



Scheme 2.

monomer **7**, and subsequent capping of unreacted amino functions with acetic anhydride. Conversion of **9** into the immobilized and fully protected thioester **11** was effected following the three-step protocol, described above, using commercially available *N*-Boc protected thiaproline **10** (see Scheme 2) in the condensation step. Subjection of **11** to trifluoromethanesulfonic acid (TFMSA) in TFA in the presence of the scavenger triisopropylsilane (TIS) led to deblocking of the Z- and Boc-groups as well as concomitant cleavage from the resin. The resin was removed by filtration and the filtrate was triturated from diethyl ether. The resulting fluffy solid was purified using RP-HPLC to give homogeneous (see trace A in Fig. 2) **4** having the expected mass ($M+H^+$: 2174.6) in 38% yield based on **6**. The tridecameric peptide **5** containing an N-terminal cysteine and the octameric C-terminal thioester peptide **2** were randomly chosen as the components in the intended two-step ligation (see Scheme 3) with the bifunctional PNA **4**. Both peptides were prepared following well established SPPS protocols, and were used without further purification. Thus, peptide **5** was readily accessible according to an Fmoc-based SPPS on Tentagel™ resin anchored to the acid labile Rink amide linker. Peptide **2** was assembled from Fmoc-glycine, immobilized on Kenner's[15] safety-catch linker, by adopting the synthesis protocol of Pessi et al.[16]

The two-step ligation of **4** with the crude peptides **2** and **5** was performed in a phosphate buffer (pH 8, 100 mM) containing the chelating agent EDTA (20 mM), guanidine hydrochloride (6 M) as the denaturating agent, and thiophenol (4%, v/v) as conjugation enhancer. The first step of the ligation process was started by the addition of **4** (1 μM) and excess **5** (5 equiv.) to the ligation buffer (400 μL). LCMS analysis, after incubation for 16 h at 20°C, showed complete conversion (see trace B in Fig. 2) of **4** into the expected ligation product **12**. The successful outcome of this step was an incentive of in situ transforming **12** into the required N-terminal cysteine derivative **3**. To this end, the pH of the solution was adjusted to pH 4 by the addition of *O*-methylhydroxylamine hydrochloride (0.5 M). After 3 h at 20°C, LCMS revealed (see trace B in Fig. 2) a quantitative conversion of **12** into **3**, having the same retention time as the starting material. It is also evident that the difference in R_f values between peptide **5** and the ligation product **3** is marginal. Consequently, purification of the crude ligation mixture by RP-HPLC afforded, as expected, adduct **3** still contaminated with peptide **5**. Ligation of impure **3** with the peptide thioester **2** (5 equiv.) under the same conditions as mentioned before proceeded uneventfully, as gauged by LCMS, to afford the ligation product **1** as well as the henicosameric peptide **13** (see trace C in Fig. 2) resulting from the ligation of **5** with **2**. Purification of the reaction mixture by RP-HPLC gave homogeneous target compound **1** (47% yield based on **4**), the identity of which was ascertained by MALDI-TOF spectrometry (calcd for $M+H^+$: 4247.60, found: 4247.54).

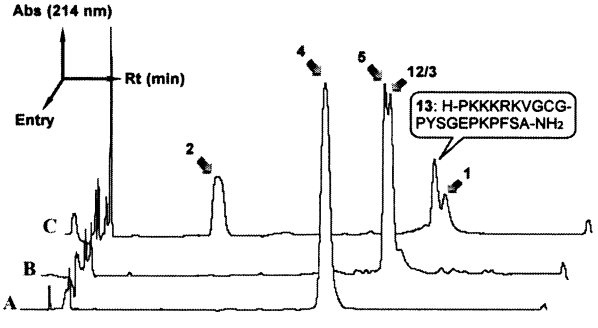
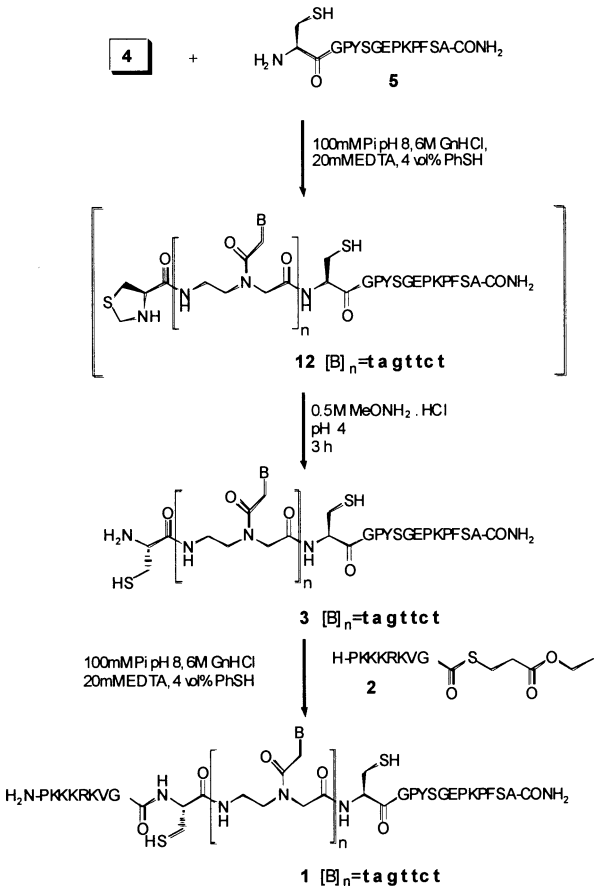


Figure 2. LCMS traces (Alltima-C18 analytical RP column; gradient 5–30% acetonitrile in 0.1% TFA over 20 min) of: (A) PNA thioester **4** in ligation buffer ($Rt = 14.5$); (B) ligation of **4** with **5** after 16 h at 20°C ($Rt_5 = 16.3$; $Rt_{12/3} = 16.5$); (C): ligation of **3** with **2** after 16 h at 20°C ($Rt_2 = 7.2$, $Rt_{13} = 17.5$, $Rt_1 = 18.0$).



Scheme 3.

The results presented in this paper show that the now readily accessible bifunctional PNA-derivative **4** may be of great value in the construction of PNA-based modular shuttles containing exposed topogenic and cell-permeable peptide sequences, which are essential elements in the delivery of PNA into the nucleus of living cells. At present we are studying in detail whether the ligation process can be optimized using the recently reported[10b] procedure of purifying peptides with an N-terminal cysteine, following a so-called reversible capture protocol. Thus, it may be expected that the disadvantage of using an excess of the N-terminal cysteine peptide in the first step of the ligation process

can be overcome by executing a reversible capture step. It is also evident that the removal of the peptide with an N-terminal cysteine will greatly facilitate the purification of the target peptide–PNA–peptide conjugates. The results of incorporating a reversible capture step in the synthesis of novel PNA-based antisense agents will be reported in due course.

Acknowledgements

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