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Synthesis of macrocyclic peptide nucleic acid derivatives via intramolecular chemical ligation

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Abstract—Macrocyclic peptide nucleic acids can be obtained by direct or indirect intramolecular cyclisation of N-terminal cysteine functionalised PNA-thioesters

Peptide nucleic acids (PNAs) are non-ionic DNA mimics that consist of an *N*-2-aminoethylglycine backbone to which the nucleobases are attached via an acetyl linker. PNAs show exceptionally tight binding and high specificity for complementary DNA and RNA sequences. The latter features, together with their resistance to nuclease and protease digestion, have led to the successful application of PNA as antisense agents in vitro.[1,2] Interestingly, Condom et al. recently[3] reported that the cyclic PNA adduct **2** (see Fig. 1), constituted by a hexameric PNA sequence and a spacer (17 atoms) tethering the N- and C-termini of the PNA, had the ability to inhibit the HIV-1 RNA dimerisation process, a crucial event in the virus development.[4] The assembly of this biologically interesting type of macrocyclic PNA construct comprised (see Fig. 1) the following two distinct steps: (i) liquid-phase synthesis of the orthogonally protected (i.e. P^1 = Troc, P^2 = Alloc and P^3 = Boc) cyclic precursor **1**, and (ii) stepwise introduction of the requisite nucleobases followed by the unmasking of the exocyclic protecting groups (i.e. benzyl and benzyloxycarbonyl) from the respective nucleobases **g** and **c**. On the basis of our earlier[5] report on the successful use of Kent's chemical ligation methodology[6] in the synthesis of PNA-peptide conjugates, we envisaged that intramolecular chemical ligation of a PNA-thioester, having an additional N-terminal cysteine unit, would be an attractive and simple route to cyclic PNA molecules. Thus, intramolecular chemical ligation of the linear cysteine-PNA-thioester **3** (see Fig. 1), obtained by solid-phase synthesis, would give access to the target cyclic PNA **4**.

In order to substantiate the concept formulated above, we first set out to prepare the non-spacer containing cyclic hexameric thymine PNA fragment **12a** (see Scheme 2). The first stage entails, as depicted in Scheme

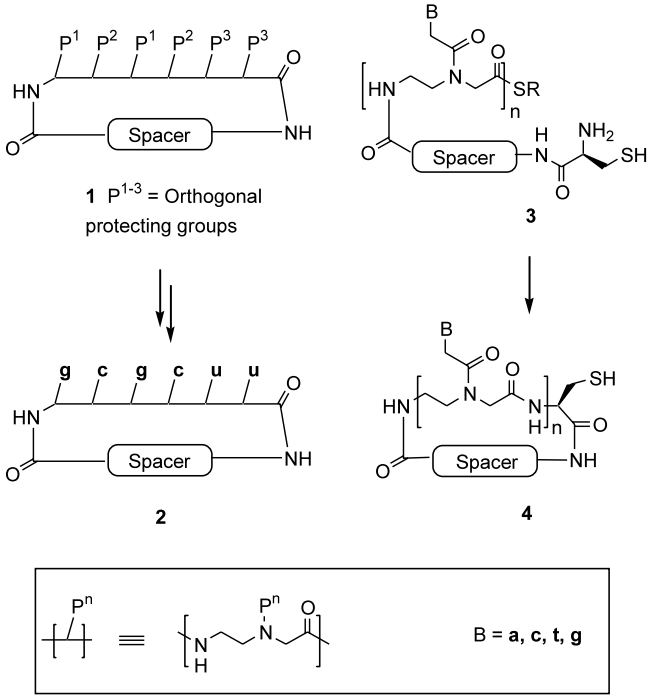

Figure 1.

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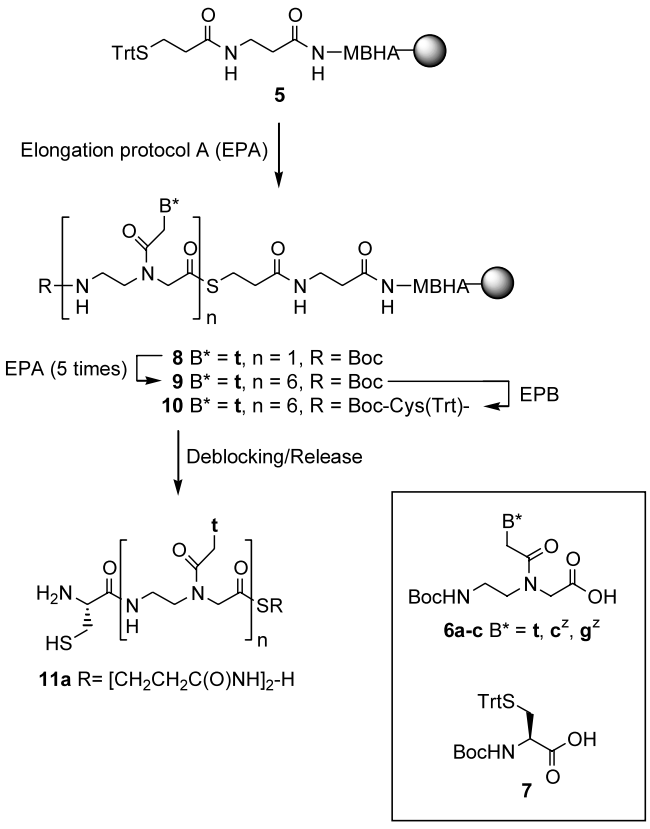
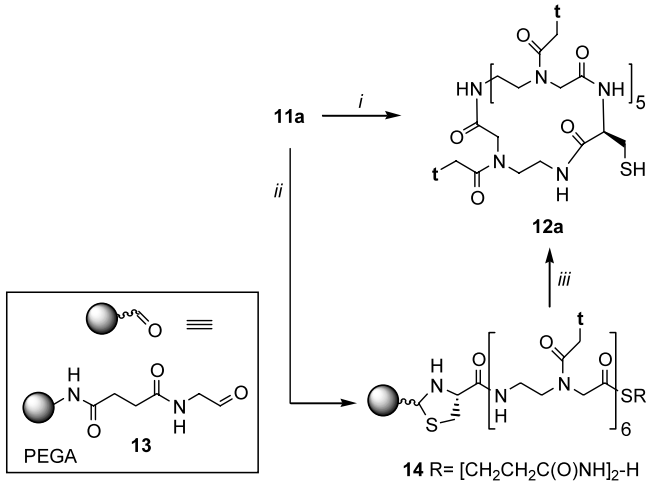
1, sequential elongation of the readily available[7] trityl-protected thiol-linker immobilised on the methylbenzhydrylamide (MBHA) resin **5** (loading capacity 0.25 mmol/g) with the *N*-Boc protected PNA and cysteine units **6a** and **7**, respectively. The advantage of using Boc- instead of Fmoc-chemistry is two-fold. First of all, elongation of **5** via Boc-chemistry is compatible with the presence of the rather base labile thioester function.[7] In addition, release from the solid support, as well as the removal of the *S*-trityl and *N*-Boc protecting groups can be effected by a simple acidolysis step. Thus, detritylation of **5** using trifluoroacetic acid (TFA) in dichloromethane followed by condensation of the thiol function with the PNA unit **6a** under the influence of HATU gave, after capping of unreacted thiol groups with acetic anhydride, the immobilised product **8**. Further elongation of **8** by repeating (5×) the same three-step protocol led to the fully protected hexameric PNA adduct **9**. Extension of the latter product with the cysteine unit **7**, under similar conditions (elongation protocol B), led to the immobilised compound **10**. Subsequent acidolysis with trifluoromethanesulfonic acid (TFMSA, 10%) in TFA gave, after trituration of the released product from cold diethylether and subsequent lyophilisation from water, the cys-PNA-thioester **11a** as a fluffy white solid, which was used without further purification in the cyclisation step outlined in Scheme 2. Thus, to a solution of crude **11a** (1 mM) in phosphate buffer (0.1 M, pH 7.6), containing the denaturating agent guanidine·HCl (6 M) and the reducing agent tris-(2-carboxyethyl)phosphine (TCEP, 0.1 M), was added the relatively mild conjugate enhancer 3-mercaptopropionic acid.[8] After 6 h at 20°C, LC/MS spectrometry revealed the presence of the cyclic target molecule **12a** as the major product. Purification by HPLC led to the isolation of homogeneous **12a**, as gauged by MALDI-TOF mass spectrometry, in a yield of 60% based on **5**.



Scheme 1. *Reagents and conditions*: EPA: 1. 50% TFA/DCM, 15 min; 2. **6a–c** (5 equiv.), HATU (4.9 equiv.), DiPEA (5 equiv.), 2,6-lutidine (7.5 equiv.), NMP, 30 min; 3. Ac_2O:2,6-lutidine:NMP, 5:6:89, v:v:v., 1 min; EPB: 1. 50% TFA/DCM, 15 min; 2. **7** (5 equiv.), HATU (4.9 equiv.), DiPEA (5 equiv.), 2,6-lutidine (7.5 equiv.), NMP, 30 min; 3. Ac_2O:2,6-lutidine:NMP, 1 min; deblocking/release: TFMSA:TIS:TFA, 10:10:80, v:v:v, 1.5 h.

At this stage, we were interested to find out whether the purity of the crude (see Fig. 2A) linear cys-PNA-thioester **11a** could be improved by the recently devised solid support mediated capture–release procedure (see Scheme 2) of Rose et al.[9] To this end, the aldehyde-functionalised PEGA resin (loading capacity 0.4 mmol/g) was added to crude **11a** in acetate buffer pH 4.3. HPLC analysis, after 6 h incubation at 20°C, revealed the absence (see Fig. 2B) of **11a**, indicating the formation of the expected immobilised thiazolidine adduct **14**. The resin was collected by filtration and washed extensively with a mixture of TFA (1%) in acetonitrile–water (1:4, v/v) to remove any uncaptured impurities. The identity of **14** was also independently corroborated by the release of **11a** from **14** under the influence of methoxylamine hydrochloride. Accordingly, a small quantity of **14** was suspended in an aqueous solution (pH 3) of methoxylamine hydrochloride (0.25 M), and left under gentle shaking for 4 h at 20°C. HPLC analysis of the supernatant showed (see Fig. 2C) the presence of a major product, the identity of which was in full accord, as gauged by LC/MS spectrometry, with the starting compound **11a**.[10] The outcome of the latter pilot experiment clearly shows that the capture–release



Scheme 2. *Reagents and conditions*: (i) ~10 equiv. $HSCH_2CH_2COOH$, 6 M guanidine·HCl, 0.1 M TCEP, 0.1 M Na_2HPO_4, pH 7.6; (ii) **13** (3 equiv.), 0.1 M NaOAc, pH 4.3, 50 mM TCEP; (iii) 1. 0.25 M $MeONH_2$·HCl, 6 M guanidine·HCl, 0.1 M TCEP, 0.1 M Na_2HPO_4, pH 3; 2. pH →7.6, add ~10 equiv. $HSCH_2CH_2COOH$.

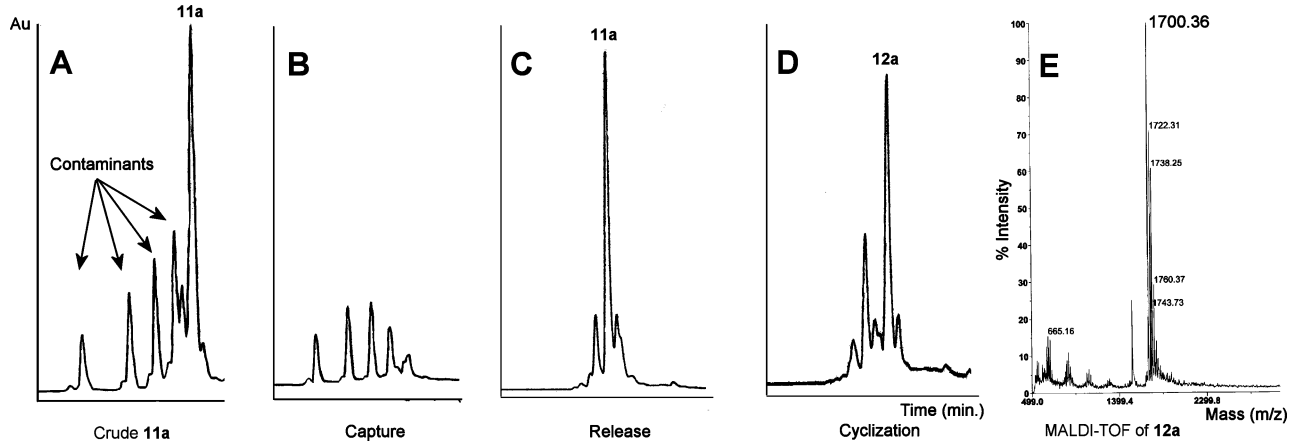
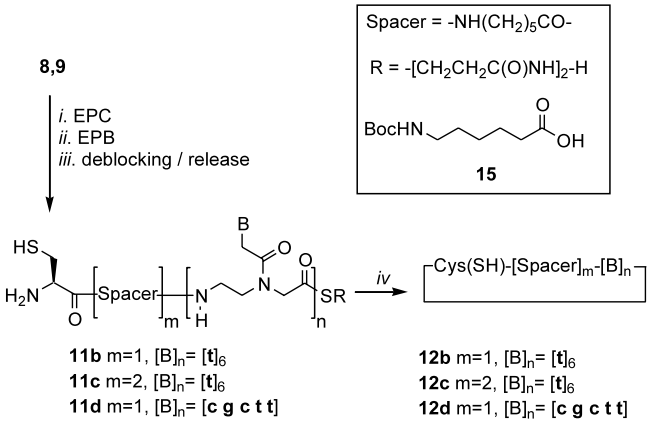


Figure 2. HPLC traces (A–D) of (A) crude **11a**, (B) supernatant of capture reaction, (C) supernatant of release mixture, (D) reaction mixture after 6 h cyclisation time and (E) MALDI TOF spectrum of purified **12a**.

protocol is not only compatible with the presence in **11a** of the rather base-labile thioester function, but also has a beneficial effect on the purity of **11a**. Apart from this, the efficiency of the capture–release process also opened the way of converting immobilised **14** into the target molecule **12a** via the following two-step, one-pot procedure. In the first step, the thiazolidine adduct **14** was suspended in the aforementioned denaturating buffer, which was adjusted to pH 3 by the addition of methoxylamine hydrochloride (0.25 M), and left under gentle shaking for 4 h at 20°C. The pH was then carefully raised to pH 7.6 with 1N sodium hydroxide followed by the addition of 3-mercaptopropionic acid (~10 equiv.) Gratifyingly, LC/MS analysis (see Fig. 2D) of the mixture, after a 6 h incubation at 20°C, showed predominant formation of the cyclic product **12a**. Purification using an Alltima C_{18} semi-preparative column and applying a gradient of acetonitrile in 0.1% TFA gave **12a**, as evidenced by MALDI-TOF (see Fig. 2E), in a comparable yield.

The successful synthesis of the relatively small cyclic PNA **12a** was an incentive to assess the efficiency of converting (see Scheme 3) the spacer (7 atoms) containing linear adducts **11b–d** into the corresponding macrocyclic products **12b–d**. The preparation of **11b**, having one spacer unit, was readily accomplished by sequential elongation of the immobilised hexameric PNA construct **9** (B*=**t**, n=6) with the N^6-Boc protected aminocaproic acid **15** and cysteine unit **7** to afford, after further processing as mentioned earlier for the elaboration of immobilised **10** (see Scheme 1), crude **11b**. Similarly, elongation of **9** (B*=**t**, n=6) with **15** (2×) and **7**, led to the isolation of crude **11c**. On the other hand, sequential elongation of **8** (B*=**t**, n=2) with the *N*-benzyloxycarbonyl (Z) protected PNA units **6b,c** yielded, after further processing as mentioned for the synthesis of **11a**, the crude heteromeric PNA **11d**.[11] Intramolecular chemical ligation of the PNA adducts **11b–d**[10] via the direct cyclisation approach gave, after purification by HPLC, the corresponding cyclic products **12b–d**[12] in an overall yield of 53, 42 and 66%, respectively. The decline in yield of the two-spacer containing cyclic product **12c** may be attributed to the increased ring size. The latter was supported by the observed relatively sluggish cyclisation of **11c** and partial hydrolysis of the intrinsically base labile thioester function.

The results presented in this paper clearly show that native chemical ligation of crude PNA, containing an N-terminal cysteine and a C-terminal thioester is a simple and straightforward route to biologically interesting macrocyclic PNA constructs. Moreover, immobilization of the cysteine unit in a crude cys-PNA-thioester adduct on an aldehyde-functionalised resin afforded, after release from the resin, the cys-PNA-thioester of high quality. Apart from this, it is also worth mentioning that the presence of a free thiol group in the cyclic products can in principle be used for the introduction of other functionalities (e.g. a fluorescent marker or cell-membrane permeable peptide). Pre-



Scheme 3. *Reagents and conditions*: (i) EPC: 1. 50% TFA/DCM, 15 min; 2. **15** (5 equiv.), HATU (4.9 equiv.), DiPEA (5 equiv.), 2,6-lutidine (7.5 equiv.), NMP, 30 min; 3. Ac_2O:2,6-lutidine:NMP, 5:6:89, v:v:v., 1 min; (ii) EPB (see Scheme 1); (iii) TFMSA/TIS/TFA 10/10/80, v/v/v, 1.5 h.; (iv) 1 mM in 6 M guanidine·HCl, 0.1 M TCEP, 0.1 M Na_2HPO_4, pH 7.6, ~10 equiv. $HSCH_2CH_2COOH$.

liminary experiments also indicated that the immobilised thiazolidine-PNA-thioester **14** showed great promise in a solid-phase synthesis of PNA having N- and C-terminal peptides which facilitate cellular uptake and delivery to the nucleus. The scope of the latter approach is currently under investigation and will be reported in due course.

Acknowledgements

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10. RP-HPLC: 5–30% B (A = 0.1% TFA in 5:95 ACN/Water, B = 0.1% TFA in 95:5 ACN/Water.), Alltima C_{18} analytical column; ESI-MS: **11a**: $[M+H]^+$: 1878.4 (calcd 1877.9); **11b**: $[M+H]^+$: 1991.4 (calcd 1991.1); **11c**: $[M+H]^+$: 2104.6 (calcd 2104.2); **11d**: $[M+H]^+$: 1719.0 (calcd 1719.8).
11. Interestingly, attempts to prepare an isomer of **11d**, containing a PNA-spacer-thioester instead of spacer-PNA-thioester, gave after deblocking the C-terminal carboxylic acid instead of the thioester. Apparently, contrary to thioesters having a nitrogen atom (peptides, PNA) or oxygen atom (using a 2-(2-aminoethoxy)ethoxy acetic acid linker (unpublished results)) in the β-position, aliphatic thioesters (carbon in β-position) are not stable towards strong acidic conditions. See also: Limura, S.; Manabe, K.; Kobayashi, S. *Org. Lett.* **2003**, *5*, 101–103.
12. RP-HPLC: 5–30% B (A = 0.1% TFA in 5:95 ACN/water, B = 0.1% TFA in 95:5 ACN/water), Alltima C_{18} analytical column; ESI-MS: **12a**: $[M+H]^+$: 1701.2 (calcd 1701.7), **12b**: $[M+H]^+$: 1815.4 (calcd 1814.8), **12c**: $[M+H]^+$: 1928.1 (calcd 1928.0), **12d**: $[M+H]^+$: 1544.0 (calcd 1543.6).