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Synthesis of thiol-modified peptide nucleic acids designed for post-assembly conjugation reactions

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Abstract—Two orthogonally protected PNA monomers were prepared having the mercaptomethyl moiety attached to the PNA backbone. These building blocks were employed in solid-phase PNA synthesis and it was shown that Boc/S-*p*-methoxybenzyl protection scheme was only satisfactory for the introduction of N-terminal thiol modification while the Fmoc/S-butylthio protected monomer proved to be amenable to elongation. The mercaptomethyl modification did not influence the thermal stability of a PNA/RNA duplex. The feasibility of PNA–PNA native ligation was demonstrated.
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1. Introduction

Peptide nucleic acids (PNAs) are achiral, uncharged DNA mimics that bind strongly to DNA and RNA in a sequence-specific manner.[1–3] PNAs are composed of repeating 2-aminoethylglycine units of which the secondary amine is connected to a nucleobase via a methylene–carbonyl linker (Fig. 1, **1**). PNAs are both chemically and biologically stable, which makes them attractive as leads for the development of gene therapeutics and as biomolecular tools.[1,4]

PNAs have been conjugated to a wide variety of ligands, such as artificial nucleases,[5] peptides,[6] intercalators[7,8] or fluorescent reporter groups[9] in order to combine the favorable properties of both entities in a single construct. In most of these conjugates the ligand is attached to either the C- or N-terminal end of the PNA. Current strategies to link ligands at various points along the PNA chain rely on replacement of a nucleobase by the desired ligand.[8,10]

We reasoned that the development of a PNA monomer in which the backbone is provided with a suitable handle would give the opportunity to connect various ligands at a predetermined position in the PNA sequence.

Over the years, a number of backbone-modified PNAs have been prepared[11] to study the effect of introducing chirality, charge or steric bulk on their physicochemical properties such as hybridization or solubility. In a very recent article, Englund and Appella[12] describe an amine-modified PNA backbone for linkage to a fluorophore. Alternatively, the application of a suitably protected thiol-modified PNA monomer (**2**) would give access to a PNA oligomer (**3**) containing a sulfhydryl group suitable for post-assembly conjugation employing well-established conjugation methods[6] (Fig. 1, **4**). Moreover, the installation of a thiol-modified PNA monomer at the N-terminus of a PNA sequence leads to a PNA with the N-terminal 1,2-aminothiol motive (**5**), which may be exploited in a chemical ligation reaction[13] with a PNA thioester (**6**)[14] to give the full length PNA (**3**) having the modified PNA monomer at the ligation site.

In this paper, we focus on the synthesis of two suitably protected thiol-modified PNA monomers (**12** and **23**, Fig. 2) and their incorporation into PNA oligomers. The position and stereochemistry (*R*-isomer and substitution at the γ-position) seemed favorable in terms of accommodation of a substituent on the basis of the NMR-structures of a PNA/R(D)NA duplex.[15] While many PNA backbone modifications have shown a deleterious effect on PNA hybridization,[16] we show that the thiol modification in **3**

Keywords: Peptide nucleic acid; Conjugation; Solid-phase synthesis.
Abbreviations: Boc, *t*-butyloxycarbonyl; Bhoc, benzhydryloxycarbonyl; DTT, dithiothreitol; Fmoc, 9-fluorenylmethyloxycarbonyl; PMB, *para*-methoxybenzyl; EDC, *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride; HATU, 2-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole hydrate; TFA, trifluoroacetic acid; NMM, *N*-methylmorpholine; TEA, triethylamine; Cbz, benzyloxycarbonyl; DIC, diisopropylcarbodiimide; DiPEA, diisopropylethylamine; TIS, triisopropylsilane; TFMSA, trifluoromethanesulfonic acid; MBHA, methylbenzhydrylamine; ma, major; mi, minor.
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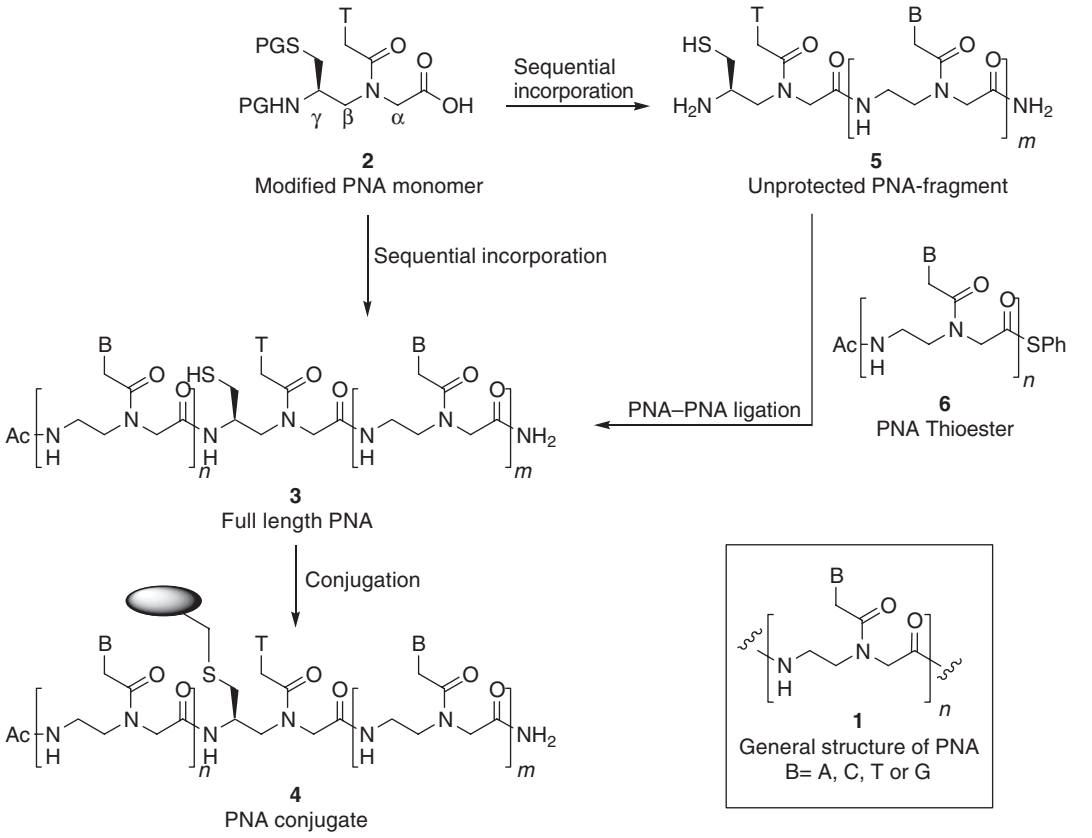


Figure 1. Synthetic strategies towards thiol-modified PNA oligomers.

hardly influences the stability of a PNA/RNA duplex. Finally, we provide data that support the assumption that the PNA–PNA ligation approach is feasible with N-terminal thymine monomers.[17]

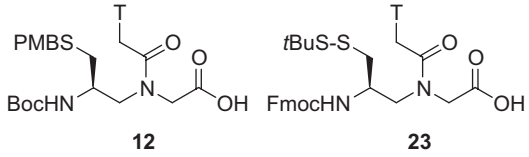
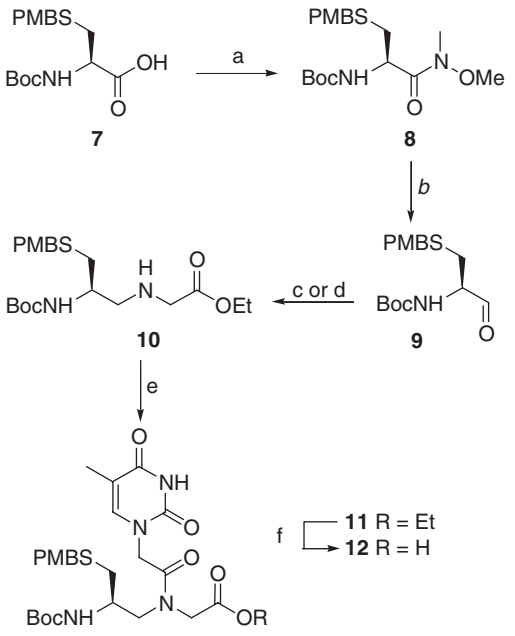


Figure 2. Target backbone-modified thymine PNA monomers.

2. Results and discussion

The protecting group pattern in PNA monomers **12** and **23** (i.e., Boc/PMB and Fmoc/S*t*Bu, respectively) facilitates the use of the two standard elongation protocols for solid-supported PNA synthesis.[18,19] The S*t*Bu group in Fmoc-protected **23** is compatible with release of the PNA oligomer from the resin and deprotection of the nucleobases (Bhoc-groups). The stability of this protecting group precludes oxidation or dimerization of the PNAs by the formation of disulfide bonds. On the other hand, the S*t*Bu group can be elegantly cleaved in situ using a water-soluble phosphine, for example during the conjugation reaction. The PMB group in **12** is stable to the conditions used for Boc-chemistry (TFA) but can be cleaved in concert with the Cbz groups on the nucleobases and concomitant cleavage from the resin.

The preparation of the Boc/PMB monomer **12** started with commercially available *N-tert*-butyloxycarbonyl-S-*p*-methoxybenzyl-protected L-cysteine **7**, which was converted into the Weinreb amide **8** in good yield (Scheme 1). Reduction of **8** with $LiAlH_4$ to give intermediate aldehyde **9** and subsequent reductive amination with glycine ethylester gave, after column chromatography, the modified PNA backbone **10** in 45% yield over the two steps. The chiral purity of **10** was ascertained (>95% ee) using chiral HPLC by comparison with the independently prepared *S*-isomer of **10**. In an attempt to increase the somewhat disappointing yield of the reductive amination by executing an aza-Wittig reaction between aldehyde **9** and readily available[20] azidoglycine ethylester was executed. However, trimethylphosphine mediated imine formation and in situ reduction with $NaCNBH_3$ afforded the modified PNA backbone **10** in a comparable yield. The final steps to the target PNA building block **12** comprised the EDC-mediated installation of the thymine nucleobase (→**11**) in 83% yield and saponification of the ethylester in **11**, which was effected quantitatively using LiOH in a mixture of methanol and water. The identity and purity of the modified PNA monomer **12** were confirmed by NMR, HPLC and mass spectrometry.

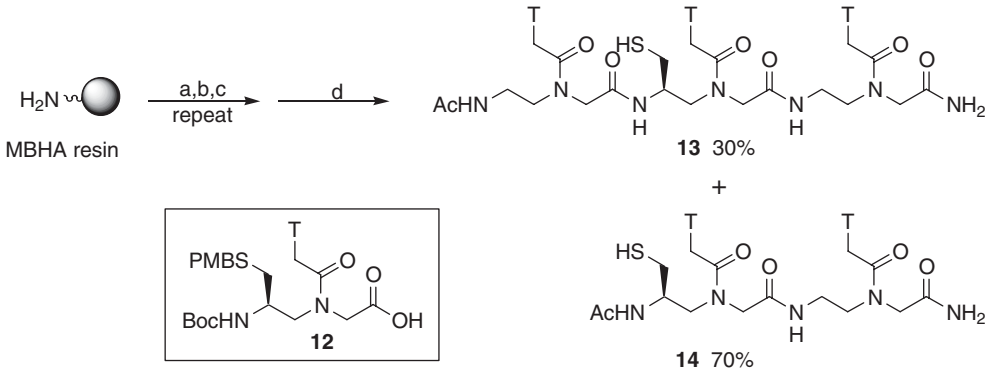


Scheme 1. Reagents and conditions: (a) MeONHMe·HCl, DIC (78%); (b) $LiAlH_4/Et_2O$; (c) glycine ethylester·hydrochloride, $NaCNBH_3$, AcOH, MeOH (45%, two steps); (d) azidoglycine ethylester and Me_3P in THF, then **9**, $NaCNBH_3$, (43%, two steps); (e) thymine-1-yl-acetic acid, EDC, DMF (85%); (f) LiOH, $MeOH/H_2O$, then Dowex-H^+ (quant.)

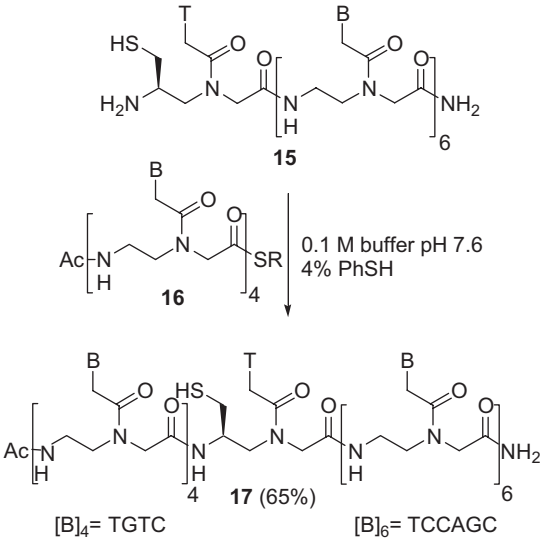
Next, the viability of the Boc/PMB-protected modified PNA monomer **12** in standard in situ neutralization protocols[19] for Boc-based PNA synthesis on the methylbenzhydrylamine (MBHA) resin was investigated (Scheme 2). Thus, after partial loading (0.25 mmol/g) of the commercially available resin with Boc-PNA(T) monomer using a HATU-mediated reaction two elongation cycles involving Boc group removal (TFA), coupling of the monomer (5 equiv) and capping were carried out. Finally, the Boc group was removed and the N-terminus was acetylated. Cleavage from the solid-support and deprotection was effected by subjection of the resin to trifluoromethanesulfonic acid in TFA and triisopropylsilane as the scavenger for 2 h. After filtration and precipitation with cold ether, the crude product was dissolved in water. LC–MS-analysis of this sample revealed the presence of two major products (30/70), which were identified by mass spectrometry to be the target trimer **13** and dimer **14**, respectively. The latter compound evidently resulted from an inefficient coupling of the third PNA monomer. Attempts to increase the trimer/dimer ratio by increasing the excess of reactants and reaction times failed.

However, the successful coupling of **12** to the N-terminus of a PNA oligomer allowed us to investigate the feasibility of the chemical ligation approach depicted in Figure 1. Reaction of the separately prepared hexameric PNA derivative **15**[14] (Scheme 3) with a slight excess of PNA thioester **16**[15] in a phosphate buffer pH 7.6 containing 4% thiophenol resulted, as judged by LC–MS (see Fig. 3), in complete[22] conversion of **15** into PNA undecamer **17**.

At this stage, the preparation of the Fmoc/S*t*Bu-monomer **23** (Scheme 4) was undertaken, following a strategy involving reductive amination of the aldehyde obtained by $LiAlH_4$ reduction of Weinreb amide **19**, with glycine-*t*-butyl ester. A similar strategy was reported[12h] to be effective in the preparation of an *N*-Fmoc-PNA backbone having a methyl substituent at the γ-carbon. Since the thio-*tert*-butyl function would not survive $LiAlH_4$ reduction, it was decided to use *N*-Fmoc-S-Trt-cysteine as the starting compound and replace the Trt-group with the S*t*Bu group in a later stage. Thus, *N*-Fmoc-S-Trt-protected L-cysteine **18** was converted to the corresponding Weinreb amide **19** in 97% yield by treatment with isobutyl chloroformate and *N*,*O*-dimethylhydroxylamine. Lithium aluminium hydride reduction gave the corresponding aldehyde, which was directly used in the next step to suppress racemization. Reductive amination with glycine *tert*-butyl ester and $NaBH_3CN$ gave, after rather tedious column chromatography, the PNA backbone **20** in 42% yield over the two steps. It is of interest to note that **20** was not stable on prolonged storage probably due to intramolecular Fmoc cleavage by the secondary amine as previously reported for the corresponding unmodified PNA backbone.[20] Reaction of the backbone **20** with thymine-1-yl-acetic acid and EDC as the coupling reagent gave the fully protected monomer **21** in 70% yield. Preferably, crude **20** was directly condensed with thymine-1-yl-acetic acid to give monomer **21** in 36% overall yield for three steps. Column chromatography purification was easier at this stage and the overall yields for the two routes were comparable.



Scheme 2. Reagents and conditions: (a) Boc-T-OH or **12**, HATU, DiPEA, DMF; (b) Ac_2O, DiPEA, DMF; (c) 50% TFA/DCM; (d) TFMSA/TFA/TIS, 10:80:10, v/v/v.



Scheme 3. Chemical ligation of two PNA oligomers. R= $[CH_2CH_2CONH]_2H$.

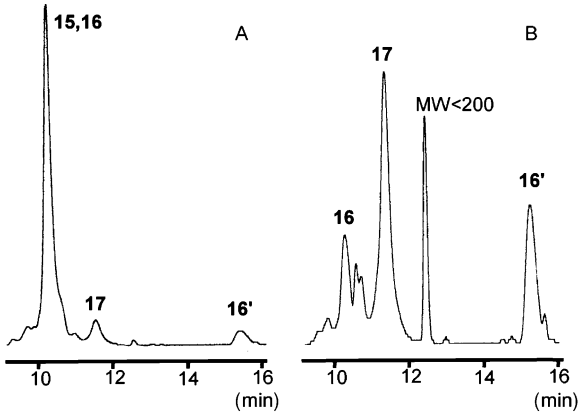
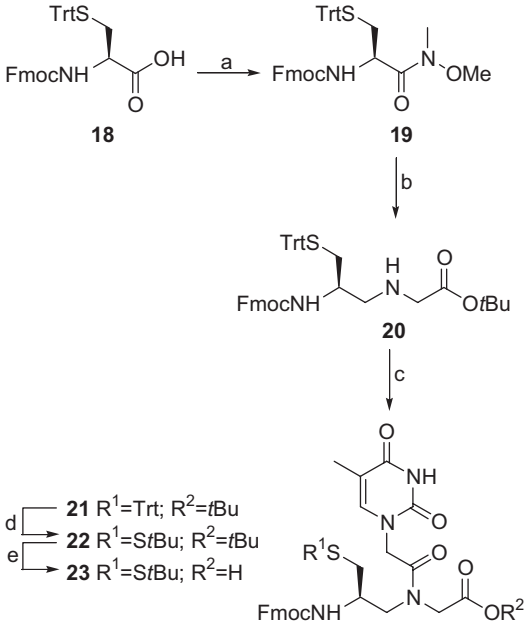


Figure 3. Parts of the HPLC traces of the ligation mixtures. A: $t=15$ min; B: $t=20$ h. **16′** is the thiophenylester of **16**; the unidentified peak (MW < 200) disappears after the addition of dithiothreitol (DTT).

Comparison of **21** with its enantiomer (independently prepared starting from *N*-Fmoc-S-Trt-D-cysteine) by chiral HPLC revealed an enantiomeric purity >95% for both enantiomers.

With the fully protected monomer **21** in hand, attempts were made to exchange the trityl protecting group for the thio-*tert*-butyl function. Initially, TFA-mediated removal of the trityl group and concomitant cleavage of the *t*-butyl ester was followed by direct reaction of the resulting intermediate with different disulfides (either the symmetrical dimer of *t*-butylthiol or the mixed dimer of *t*-butylthiol and 2-pyridyl-thiol). However, these reactions gave complex mixtures (according to LC–MS analysis) and the products were not separable by column chromatography. Fortunately, exchange of the trityl group for the thio-*tert*-butyl function could be effected in a single step by treatment of **21** with an excess of iodine and *t*-butylthiol in DCM in the presence of pyridine in 86% yield. The pyridine was added to prevent the

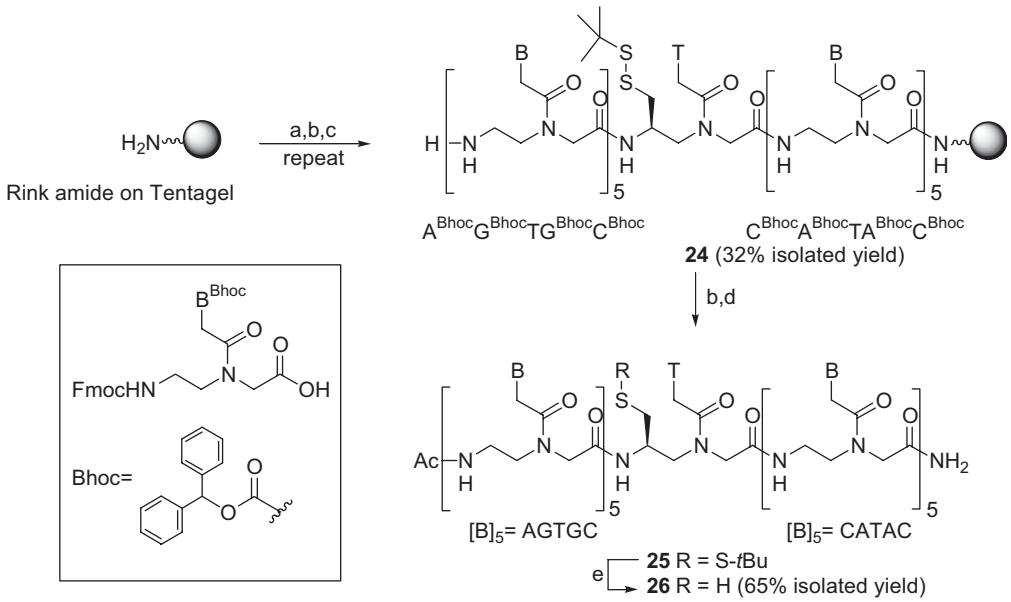


Scheme 4. Reagents and conditions: (a) 1. isobutyl chloroformate, NMM, THF, −20 °C; 2. *N*,*O*-dimethylhydroxylamine hydrochloride, TEA, DMF, −20 °C, 97%; (b) 1. $LiAlH_4$, Et_2O, 0 °C; 2. Gly-O*t*Bu·HCl, $NaBH_3CN$, MeOH, 3 Å sieves, 42% (two steps); (c) thymine-1-acetic acid, EDC·HCl, 36% (three steps); (d) 2-methyl-2-propanethiol, I_2, pyridine, DCM, 86%; (e) 90% TFA/DCM, quant.

otherwise observed premature cleavage of the *t*-butyl ester. Finally, cleavage of the ester in **22** with TFA afforded the desired monomer **23** in quantitative yield.

The incorporation of PNA monomer **23** into a PNA oligomer (AGTGCT*CATAC where T* represents the modified monomer) was carried out with an automated synthesizer using TentaGel® resin loaded with the Rink amide linker (Scheme 5). Elongation (→**24**) was effected by applying the standard Fmoc-synthesis protocols supplied by the manufacturer (using commercially available Fmoc-PNA(B^{Bhoc})-OH monomers, **23** and HATU/DiPEA-lutidine as the coupling reagents). After standard deprotection and release from the resin (95% TFA, TIS, water), the S*t*Bu protected PNA **25** was precipitated from ether. LC–MS analysis of the crude mixture revealed the presence of a single major product having the expected mass, thus indicating that coupling and subsequent elongation of the modified monomer could be accomplished using standard Fmoc-based PNA chemistry. The S*t*Bu group in crude **25** could be removed efficiently by treatment with a solution of tris(2-carboxyethyl)phosphine (TCEP) in buffer (pH 6). PNAs **25** and **26** were readily purified by HPLC and used to investigate the effect of the thiol-modification on the duplex formation.

The hybridization properties of the modified PNAs **25** and **26** with complementary, antiparallel RNA were examined using variable-temperature UV. Comparison of the melting temperatures showed that the backbone modification (i.e., CH_2SH or CH_2SStBu) did not significantly affect the stability of the duplex (Table 1).



Scheme 5. Reagents and conditions: (a) Fmoc/Bhoc-PNA monomer or **23**, HATU, DiPEA, lutidine, DMF/NMP; (b) Ac_2O, lutidine, NMP; (c) 20% piperidine, DMF; (d) TFA/water/TIS 95:2.5:2.5, 2 h; (e) 50 mM tris-(2-carboxyethyl)phosphine, pH 6.

Table 1. Melting temperatures of the (un)modified PNAs with complementary, antiparallel RNA

	T_m value (°C)
RNA/Ref PNA	67.2
RNA/**25**	68.2
RNA/**26**	67.1

The given T_m value is the average of three independent measurements. RNA (5′→3′): AUU UAA GAG UAU GAG CAC UAU CGAA; Ref PNA (N→C): AGTGCTCATAC.

3. Conclusion

Orthogonally protected thiol-modified PNA monomers **12** and **23** were prepared and applied in standard PNA synthesis protocols using Boc (**12**) and Fmoc (**23**) chemistry, respectively. The latter compound proved to be superior in terms of the yield of chain elongation after coupling of the modified monomer. It was demonstrated that the thiol-modification in synthesized undecamer PNAs **25** and **26** did not notably affect the hybridization properties with complementary RNA. The here presented methodology is a valuable asset for future conjugation of PNAs with a variety of ligands such as artificial RNA-nucleases for the sequence-selective degradation of the target RNA.[5b] Finally, pilot experiments suggested that a PNA having a modified thymine monomer at the N-terminus can be used for the ligation with PNA thioesters. This procedure[17] may be implemented in the preparation of cyclic PNAs[14b,23] as well as PNAs of unprecedented lengths.

4. Experimental

4.1. General

All reagents were used as received.

Analytical LC–MS was conducted on a JASCO system using an Alltima C_{18} analytical column (5 μ particle size, flow: 1.0 mL/min). Absorbance was measured at 214 and 254 nm. Solvent system: A: 100% water, B: 100% acetonitrile, C: 0.5% TFA. Gradients of B in 10% C were applied over 15 min unless otherwise stated. Mass spectra were recorded on a Perkin Elmer Sciex API 165 equipped with a custom-made Electrospray Interface (ESI) or for HR-MS on a LTQ-FT (Thermo Electron). Purifications were conducted on a BioCAD 'Vision' automated HPLC system (PerSeptive Biosystems, Inc.), equipped with a semi-preparative Alltima C_{18} column (5 μ particle size, running at 4 mL/min). Solvent system: A: 100% water, B: 100% acetonitrile, C: 1% TFA. Gradients of B in 10% C were applied over 3CV unless otherwise stated. A TitroLine alpha machine or Merck Universal indikator pH 1–10 pH paper was used to measure the pH of buffers.

NMR spectra were measured on Bruker AC200, AC300, AV400 or AV600 spectrometers. Chemical shifts are given in ppm, relative to the signal of the internal standard tetramethylsilane.

IR spectra were recorded on a Shimadzu FTIR-8300 spectrophotometer, $[\alpha]_D$ values were determined using a Propol Automatic Polarimeter and melting points were determined using a Buchi Schmeltzpunkt Bestimmungsapparat.

4.1.1. Boc-L-Cys(PMB)-N(Me)OMe (8). *N-tert*-Butyloxycarbonyl-S-*p*-methoxybenzyl-L-cysteine (17.07 g, 50 mmol) was dissolved in dry DMF (200 mL). To this solution were added subsequently molecular sieves, 1.6 equiv of HOBt (10.8 g), 1.6 equiv of *N*,*O*-dimethylhydroxylamine·HCl (7.76 g), 1.6 equiv of DiPEA (13.6 mL) and 2 equiv of DIC (15.5 mL). The mixture was stirred for 1 h at room temperature. TLC (eluent: EtOAc/PE, 1:1, v/v, containing 0.5% triethylamine) indicated the nearly complete conversion of the starting compound into a higher running product (R_f=0.55). The solution was filtered and evaporated to dryness. The residue was redissolved in EtOAc and

transferred into a separatory funnel. The organic layer was washed with 10% $KHSO_4$, 10% $NaHCO_3$, water and finally with brine. The organic layer was dried with $MgSO_4$, filtered and evaporated to a small volume. Silica was added and the mixture was evaporated to dryness. The solid residue was applied onto a silica column and eluted with a mixture of EtOAc/PE, 1:1, v/v, containing 0.5% Et_3N. The fractions containing the target compound were collected and evaporated to yield 15 g (39 mmol, 78%) of the product as a colorless oil.

^{1}H NMR (200 MHz, $CDCl_3$): δ 7.25, 7.22 (2×2H, CH arom.), 5.38 (1H, d, J=8.7 Hz, NH Boc), 4.88 (1H, br s, CH_α), 3.78, 3.74, 3.70 (8H, 3×s, MeO PMB, CH_2 PMB, MeO–N), 3.2 (3H, s, CH_3–N), 2.85–2.56 (2H, m, CH_2 Cys), 1.45 (9H, s, *t*Bu). ^{13}C NMR ($CDCl_3$): δ 171.0 (C=O amide), 158.1 (Cq. PMB), 154.8 (C=O Boc), 129.6 (CH arom.), 129.3 (Cq. PMB), 113.4 (CH arom.), 79.0 (Cq. *t*Bu), 61.0 (MeO), 54.7 (MeO), 49.0 (CH), 35.1, 32.8 (2×CH_2), 31.6 (CH_3), 27.8 (*t*Bu). ES MS (found/calculated): 385.4/385.5 $(M+H)^+$. $[\alpha]_D^{23}$ −19.2 (*c* 1 in $CHCl_3$) (L-isomer). IR (cm^{-1}): 1705.0, 1654.8, 1508.2, 1245.9, 1164.9, 729.0.

4.1.2. (*R*) Boc-Cys(PMB) backbone ethyl ester (10).

By reductive amination:

Compound **8** (1.85 g, 4.8 mmol) was weighed in a dried 50 mL round bottom flask, co-evaporated with DCE (2×), dissolved in freshly distilled diethylether (12 mL) and put under a nitrogen gas atmosphere. Next, the solution was cooled to −30 °C. A suspension of 1.2 equiv $LiAlH_4$ (219 mg) in freshly distilled diethylether (12 mL) was added dropwise under streaming nitrogen gas. The reaction mixture was allowed to warm to 0 °C over 20 min and analyzed with TLC, which revealed complete conversion of the starting compound (the starting compound colors blue when the TLC is charred with molybdenum, whereas the product gives a yellow coloring). The mixture was then cooled to −30 °C and a 10% solution of $KHSO_4$ (20 mL) was carefully added to quench the excess of $LiAlH_4$. The acidic mixture was allowed to warm to room temperature and transferred into a separatory funnel. The organic layer was washed three times with 10% $KHSO_4$, and one time with brine, dried ($MgSO_4$), filtered and evaporated to yield a white foam (1.56 g, 4.8 mmol) of the crude aldehyde **9**. The aldehyde was co-evaporated (2×) with DCE and dissolved in dry methanol (20 mL). 1 equiv (672 mg, 4.8 mmol) of glycine ethyl ester hydrochloride and molecular sieves were added and the reaction was stirred under a nitrogen atmosphere. 2 Equiv of $NaCNBH_3$ (605 mg, 9.6 mmol) were added and the mixture was stirred overnight at room temperature. TLC analysis (eluent: EtOAc/PE, 2:1, v/v containing AcOH (0.5%)) indicated (besides several byproducts) one major spot (R_f=0.2). The reaction mixture was filtered and evaporated to dryness. The residue was taken up in EtOAc, washed with water and brine, dried with $MgSO_4$, filtered and evaporated under reduced pressure. The residue was applied onto a silica gel column and was eluted with a gradient of EtOAc in PE (1/1→3/1, v/v) to yield the title compound as a colorless oil. (852 mg, 2.1 mmol, 45%).

By aza-Wittig-reaction:

To a cooled (0 °C) solution of azidoacetic acid ethyl ester[21] (1.66 mmol, 214 mg) in freshly distilled THF (6 mL) was added under an argon atmosphere trimethylphosphine (2 mL, 1 M in toluene). The reaction mixture was allowed to warm to room temperature and molecular sieves (3 Å) were added. Crude aldehyde **9** (1.99 mmol, 647 mg) was co-evaporated with DCE (2×) and dissolved in freshly distilled THF (6 mL). The two solutions were combined and stirred for 30 min. Next, 1.5 equiv $NaCNBH_3$ (2.99 mmol, 186 mg) in MeOH (2 mL) was added. TLC analysis (eluent: EtOAc/PE 2:1, v/v containing AcOH (0.5%)), after 20 h, indicated the formation of a main product. The reaction mixture was filtered and concentrated in vacuo. The residue was taken up in EtOAc, washed with 10% $NaHCO_3$, dried ($MgSO_4$), filtered and evaporated under reduced pressure. The residue was applied onto a silica column, which was eluted with a gradient of EtOAc in PE 1/1→3/1, v/v) to yield the title compound as a colorless oil in 35% (354 mg, 0.86 mmol).

^{1}H NMR (200 MHz, $CDCl_3$): δ 7.26 (2H, d, J=8.8 Hz, 2× CH arom. PMB), 6.84 (2H d, J=8.8 Hz, 2×CH arom. PMB), 5.22 (1H, br s, NH Boc), 4.18 (2H, q. J=7.3 Hz, CH_2 Et), 3.76 (3H, s, MeO PMB), 3.68 (2H, s, CH_2 PMB), 3.37 (2H, CH_2), 2.76–2.47 (5H, m, 2×CH_2, CH), 1.94 (1H, s, NH), 1.45 (9H, s, *t*Bu), 1.26 (3H, t, J=7.3 Hz, CH_3 Et). ^{13}C NMR ($CDCl_3$): δ 172.0 (C=O ester), 158.1 (C=O Boc), 155.2, 129.7 (2×Cq. PMB), 129.6, 113.5 (2×CH arom. PMB), 78.9 (Cq. *t*Bu), 60.3 (CH_2 Gly), 54.8 (MeO, PMB), 51.0, 50.5 (2×CH_2), 49.4 (CH), 35.6, 33.6 (2×CH_2), 28.1 (CH_3 *t*Bu), 13.9 (CH_3 Et). HR-MS (found/calculated) $(M+H)^+$: 413.2089/413.2105. $[\alpha]_D^{23}$ +12.6 (*c* 1 in $CHCl_3$) (*R*-isomer). IR (cm^{-1}): 1735.8, 1701.1, 1512.1, 1242.1, 1164.9, 1029.9, 732.9.

The synthesis of the *S*-isomer of **10** was carried out in an identical manner to that described above starting from the D-isomer of cysteine. Spectroscopic data (NMR) were identical. $[\alpha]_D^{23}$ −10.8 (*c* 1 in $CHCl_3$) (*S*-isomer).

4.1.3. (*R*) Boc-Cys(PMB) thymine monomer ethyl ester (11).

Compound **10** (284 mg, 0.69 mmol) was dissolved in dry DMF (3.5 mL). 1.5 Equiv of thymin-1-yl-acetic acid (188 mg) and 1.5 equiv of EDC (199 mg) were added and the mixture was stirred for 30 min. TLC (EtOAc/PE, 3:1, v/v) indicated complete conversion of the starting compound into a lower running product (R_f=0.6). The mixture was evaporated and the residue was taken up in EtOAc. The EtOAc-layer was washed subsequently with 10% $KHSO_4$, 10% $NaHCO_3$, water and brine, dried ($MgSO_4$), filtered and evaporated in vacuo. The crude product was purified further by column chromatography using silica gel and a gradient of EtOAc in PE (75→100%, v/v), which afforded the title compound as a white powder (0.59 mmol, 340 mg) in 85% yield.

^{1}H NMR (200 MHz, two rotamers, $CDCl_3$): δ 7.28 (2H, d, J=8.8 Hz, 2×CH arom. PMB), 7.01 (1H, s, CH thymine), 6.86 (2H, d, J=8.8 Hz, 2×CH arom. PMB), 4.30–4.07 (4H, m, CH_2 Et, CH_2), 3.78 (3H, s, MeO PMB), 3.73–3.62 (5H, m, CH, 2×CH_2), 3.51 (2H, br s, CH_2 Gly), 2.79–2.54 (2H, m, CH_2 Cys), 1.90 (mi), 1.91 (ma) (3H, 2×s, CH_3 thymine), 1.43 (mi), 1.41 (ma) (9H, 2×s, *t*Bu), 1.31 (mi), 1.28 (ma) (3H, 2×t, J=7.1 Hz, CH_3 Et). ^{13}C NMR ($CDCl_3$): δ 168.8 (C=O ester), 167.3 (C=O amide), 164.4 (C4 thymine),

158.4 (C=O Boc), 155.3 (Cq. PMB), 151.0 (C2 thymine), 141.1 (C6 thymine), 129.7 (CH arom. PMB), 129.5 (Cq. PMB), 113.7 (CH arom. PMB), 110.2 (C5 thymine), 79.5 (Cq. *t*Bu), 61.8 (mi), 61.0 (ma) (CH$_2$COOEt), 54.9 (MeO PMB, 50.3 (CH$_2$), 49.0 (CH), 48.4, 47.4, 35.7, 33.0 (4×CH$_2$), 28.0 (CH$_3$ *t*Bu), 13.8 (CH$_3$ Et), 12.0 (CH$_3$ thymine). HR-MS (found/calculated) $(M+H)^+$: 579.2521/579.2483. $[\alpha]_D^{23}$ −8.0 (*c* 1 in CHCl$_3$). Mp: 74–78 °C. HPLC: 50–90% B, t_R=3.7 min (single peak). IR (cm^{-1}): 1666.4, 1512.1, 1465.8, 1242.1, 1164.9, 910.3, 725.2.

4.1.4. (*R*) Boc-Cys(PMB)-thymine-monomer (12). Compound **11** (652 mg, 1.13 mmol) was suspended in a 2/1 (v/v) mixture of MeOH and water (8 mL) LiOH (3 equiv, 68 mg) was added and the reaction mixture was stirred for 15 min, during which the compound dissolved. TLC analysis (3:1 EtOAc/PE, v/v) indicated the disappearance of the starting compound and the formation of a product running near the baseline. The mixture was acidified (to pH 5) by the addition of Dowex-H$^+$ and filtered. The resin was washed with a mixture of water–MeOH (1/1, v/v). The combined filtrates were evaporated to afford the pure title compound as a white foam in near quantitative yield (606 mg, 1.1 mmol).

^{1}H NMR (200 MHz, two rotamers, CD$_3$OD): δ 7.19 (2H, d, J=8.8 Hz, 2×CH arom. PMB), 7.12 (1H, s, C6 thymine), 4.1–3.4 (10H, m, 3×CH$_2$, CH, MeO (3.66)), 3.21 (2H, s, CH$_2$) 2.49–2.35 (2H, m, CH$_2$ Cys), 1.78 (3H, s, CH$_3$ thymine), 1.36 (mi), 1.35 (ma) (9H, 2×s, *t*Bu). ^{13}C NMR (methanol-d_4, 75 MHz): δ 172.0 (C=O acid), 170.0, 169.5 (C=O amide), 166.7 (C4 thymine), 159.9, 159.8 (C=O Boc), 157.5 (Cq. PMB), 152.6 (C2 thymine), 143.3, 143.2 (C6 thymine), 131.3 (Cq. PMB), 131.0, 114.8 (2×CH arom. PMB), 110.9, 110.8 (C5 thymine), 80.5, 80.2 (Cq. *t*Bu), 55.6 (MeO PMB), 51.9, 50.8 (2×CH$_2$), 50.5, 50.2 (CH), 49.1, 36.6, 28.7 (3×CH$_2$), 28.7 (CH$_3$ *t*Bu), 12.3 (CH$_3$ thymine). ES MS (found/calculated) $(M+H)^+$: 551.6 (551.2). HPLC: 5–70% B, t_R=15.9 min (single peak). IR (cm^{-1}): 3355.9 (broad), 1658.7, 1242.1, 1164.9. $[\alpha]_D^{23}$ −80 (*c* 1 in CHCl$_3$). Mp=120–122 °C.

4.1.5. Modified PNA oligomer 13. PNA-synthesis was carried out manually on a 10 μmol scale (15 mg of MBHA resin 0.66 mmol/g). The Boc-protected PNA thymine monomer was pre-acivated for 1 min with HATU (4.9 equiv) and DiPEA (10 equiv) in DMF (0.5 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$_2$O/2,6-lutidine/NMP, 5:6:89, 2 mL). The resin was washed with DMF and DCM. Boc deprotection was effected by a 15 min treatment with 50% TFA/DCM (2 mL). After thorough washing with DCM and DMF, the coupling procedure was repeated using monomer **12**. The resulting resin was used in the third coupling step (i.e., Boc deprotection and coupling with the Boc-protected thymine monomer to give the immobilized trimer).

The Boc group in the immobilized trimeric compound was removed by treatment with 50% TFA/DCM (2 mL) for 15 min, followed by extensive washing with DCM and DMF. After capping (see above) the resin was washed with DMF and DCM and dried.

Deprotection/cleavage: the resin was 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 streaming argon. The mixture was kept at 0 °C for 5 more minutes and was then allowed to warm to room temperature and shaken for 1.5–2 h. The suspension was filtered into cold diethylether (30 mL) and the resin was washed with neat TFA (2×1 mL). The resulting suspension was centrifuged and the diethylether layer was decanted. The precipitate was washed with diethylether and after centrifugation, the ether was removed and the precipitate was dissolved in water and purified by semi-preparative HPLC.

Compound **13**: LC–MS: 5–35% B, t_R=10.0 min. ES MS (found/calculated) $(M+H)^+$: 904.2 (904.3).

4.1.6. PNA derivative 15. The synthesis was performed manually on a 10 μmol scale using the Rinkamide-TentaGel® resin (loading capacity 0.22 mmol/g). A single elongation cycle consisted of the following three steps: Fmoc deprotection: wash with NMP, 20% piperidine in DMF (5 min), NMP washes; elongation: DMF washes, 5 equiv of monomer (Fmoc/Bhoc-protected PNA monomers or **12** in the final coupling), 4.9 equiv of HATU, 10 equiv of DiPEA, in DMF (~0.1 M monomer concentration), 1 min preactivation, 30 min coupling time, DMF washes; capping: 5/6/89 Ac$_2$O/lutidine/DMF, 2 min. Deprotection/release from the resin was effected in a two-step procedure, to prevent capture of the released benzhydryl cation by the free thiol group.[24] The resin was suspended in a mixture of TFA (1.6 mL) and TIS (0.2 mL) and shaken for 1.5 h. The mixture was then cooled to 0 °C and TFMSA (0.2 mL) was added slowly and kept at 0 °C for another 5 min. The mixture was removed from the ice-bath and shaken for 1 h at room temperature. After filtration, the filtrate was precipitated from cold diethylether (~35 mL) and centrifuged. The residue was washed again with cold diethylether and centrifuged. The remaining crude product was dissolved in 1 mL of 0.1 M NaOAc in water–acetonitrile (1/1, v/v) and the pH was adjusted to 6. PEGA-aldehyde resin[25] (250 mg, 0.1 mmol, 10 equiv) was added and the mixture was shaken for 15 h. The resin was collected by filtration and thoroughly washed with acetonitrile–water (1/1, v/v). The resin was then suspended in a 0.2 M solution of methoxylamine·hydrochloride (1 mL, pH 3) and left for 8 h at room temperature. The resin was removed by filtration and washed with 1% TFA (1 mL total). The collected filtrates were applied to a ready desalting step using semi-preparative HPLC column chromatography (6–40% over 3 CV).

Compound **15**: LC–MS: 5–35% B, t_R=10.3 min. ES MS (found/calculated) $(M+H)^+$: 1917.6 (1916.9), $(M+2H)^{2+}$: 958.8 (959.0), $(M+3H)^{3+}$: 639.6 (639.6).

4.1.7. PNA thioester 16. PNA thioesters were prepared manually on 10 μmol scale on the TrtSCH$_2$CH$_2$CO-βAla-MBHA-resin[26] using slightly modified protocols as described previously.[14c] In brief, PNA elongation was accomplished as follows: Boc-deprotection: 15 min treatment with 50% TFA/DCM; elongation: 5 equiv monomer, 4.9 equiv HATU, 10 equiv DiPEA, 0.1 M in DMF, 1 min

preactivation, 30 min coupling time; cap: 5/6/89 Ac_2O/lutidine/DMF, 2 min. Cleavage/deprotection: 2 mL TFMSA–TIS–TFA (10/10/80, v:v), 1.5 h. Precipitation in cold diethylether (~30 mL) and centrifuging gave the crude thioester, which was purified by ready RP-HPLC.

Compound **16**: LC–MS: 5–35% B, t_R 10.1 min. ES MS (found/calculated) $(M+H)^{+}$: 1293.8 (1293.5), $(M+2H)^{2+}$: 647.6 (647.3).

4.1.8. PNA–PNA ligation→17. A stock solution of (120 μL) **15** (2 mM, determined by measuring A_{260} units) in ligation buffer containing 6 M guanidine HCl, 0.1 M Na_2HPO_4, 0.1 M TCEP, pH 7.6 was mixed with 120 μL of a solution of **16** (4.6 mg/mL; 1.8 equiv) in the same ligation buffer. Thiophenol (10 μL, 4%, V) was added and the mixture was vortexed for a few seconds and shaken for 20 h at ambient temperature. 5 mg of DTT was added and after 15 min the mixture was diluted with 1% TFA to a total volume of 1 mL and purified by RP-HPLC (6–29% B over 3 CV).

Compound **17**: Yield 0.5 mg (65%, determined by A_{260} units) LC–MS: 5–35% B, t_R 11.5 min. ES MS (found/calculated): $(M+2H)^{2+}$: 1517.6 (1517.5), $(M+3H)^{3+}$: 1011.8 (1012.0), $(M+4H)^{4+}$: 759.4 (759.2).

4.1.9. Fmoc-L-Cys(Trt)-N(CH_3)OCH_3 (19). *N*-Fmoc-L-Cys(Trt)-OH (5.86 g, 10 mmol) was dissolved in THF (60 mL), *N*-methylmorpholine (1.21 mL, 11 mmol) was added and the solution was cooled to −20 °C. Isobutylchloroformate (1.30 mL, 10 mmol) was added and the solution was stirred 30 min at the same temperature. Triethylamine (1.53 mL, 11 mmol) was added and then a solution of *N*,*O*-dimethylhydroxylamine hydrochloride (1.07 g, 11 mmol) in DMF (25 mL). The solution was stirred for 30 min at −20 °C and then 1 h at room temperature Then the solvent was removed in vacuo, the residue was taken up in EtOAc (200 mL), which was washed with 0.5 M HCl (100 mL). The aqueous layer was extracted with EtOAc (2×100 mL). The combined organic layers were dried ($MgSO_4$) and evaporated under reduced pressure. Compound **19** was isolated after column chromatography (PE/EtOAc, 1:1, R_f=0.58) as a white foam. Yield: 97%; ^{1}H NMR (600 MHz, $CDCl_3$): δ 2.46 (1H, dd, *J*=8.1, 12.2 Hz, C*H*HS), 2.62 (1H, dd, *J*=4.5, 12.3 Hz, CH*H*S), 3.16 (3H, s, NCH_3), 3.63 (3H, s, OCH_3), 4.22 (1H, t, *J*=7.2 Hz, C*H*CH_2O), 4.31–4.44 (2H, m, CHCH_2), 4.78–4.82 (1H, m, CHCO), 5.40 (1H, d, *J*=9.0 Hz, NH), 7.18–7.77 (23H, m, H_{ar}). ^{13}C NMR ($CDCl_3$): δ 32.06 (NCH_3), 33.94 (CH_2S), 47.02 (*C*HCH_2O), 50.13 (*C*HCH_2N), 61.54 (OCH_3), 66.84 (*C*($Ph)_3$), 67.03 (CH*C*H_2N), 119.89, 125.16, 126.74, 127.03, 127.64, 127.91, 129.53 (CH_{ar}), 141.19, 143.71, 143.85, 144.36, 155.74, 170.59 (C_{ar}, CO). HR-MS (found/calculated): 646.2756/646.2740 $(M+Na)^{+}$. $[\alpha]_D^{23}$ −13.6 (*c* 1 in $CHCl_3$). IR (cm^{-1}): 2360.7, 2350.4, 1716.5, 1654.8, 1245.9, 1033.8.

D-Isomer of **19**:

The synthesis was carried out in an identical manner to that described above. Spectroscopic data were identical. Yield: 92%. $[\alpha]_D^{23}$ +13.0 (*c* 1 in $CHCl_3$)

4.1.10. (*R*) Fmoc-Cys(Trt)-thymine monomer *t*Bu ester (21). Weinreb amide (**19**, 2.00 g, 2.93 mmol) was dissolved in dry Et_2O (25 mL) and the solution was cooled to 0 °C. $LiAlH_4$ (0.139 g, 3.66 mmol) was added in one portion and the reaction mixture was stirred for 10 min at 0 °C. The reaction was quenched by careful addition of 0.5 M HCl (50 mL) and the mixture was allowed to warm to room temperature The organic phase was diluted with Et_2O (50 mL) and the phases were separated. The aqueous phase was extracted with Et_2O (2×50 mL). The combined Et_2O layers were washed with 0.5 M HCl (2×50 mL), dried ($MgSO_4$) and evaporated under reduced pressure to give a foam, which was re-dissolved in MeOH (35 mL). Glycine *tert*-butyl ester HCl (0.982 g, 5.86 mmol) was dissolved in MeOH (15 mL) and added. 3 Å sieves and $NaBH_3CN$ (0.552 g, 8.79 mmol) were added and pH was checked (pH 6). The reaction mixture was stirred overnight before the solvent was evaporated and the residue was re-dissolved in EtOAc (150 mL). The organic phase was washed with $NaHCO_3$ (2×50 mL), dried ($MgSO_4$) and evaporated in vacuo to give crude **20** as a foam, which was used directly in the next reaction.

Compound **20** could be isolated with column chromatography (PE/EtOAc, gradient 2:1→1:1), but proved to be unstable. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.45 (9H, s, C($CH_3)_3$), 2.31–2.73 (4H, m, CH_2S, CHCH_2N), 3.18 (1H, d, *J*=2.2 Hz, $NHCH_2CO$), 3.42–3.70 (1H, m, C*H*CH_2N), 4.20–4.24 (1H, m, C*H*CH_2O), 4.34–4.41 (2H, m, CHCH_2O), 5.13 (1H, d, *J*=8.0 Hz, NH), 7.19–7.39 (23H, m, H_{ar}). ^{13}C NMR ($CDCl_3$): δ 27.90 (C(*C*$H_3)_3$), 34.24 (CH_2S), 47.04 (*C*HCH_2O), 50.10 (*C*HCH_2N), 51.31 (CH*C*H_2N), 66.30, 66.63 (*C*($Ph)_3$, CH*C*H_2O), 81.00 (*C*($CH_3)_3$), 119.73, 124.97, 126.55, 126.85, 127.46, 127.76, 129.40 (CH_{ar}), 141.08, 143.75, 144.41, 155.69, 171.40 (C_{ar}, CO).

Crude PNA backbone **20** obtained in the previous step, was dissolved in DMF (20 mL). Thymine-1-acetic acid (0.567 g, 3.08 mmol) was added and the mixture was stirred until the acid was dissolved. Then EDC (0.562 g, 2.93 mmol) was added and the reaction mixture was stirred overnight at room temperature Then the solvent was evaporated under reduced pressure and the residue was re-dissolved in EtOAc (150 mL). The organic layer was washed with 0.5 M HCl (2×50 mL), satd aq $NaHCO_3$ (50 mL) and brine (50 mL), dried ($MgSO_4$) and evaporated in vacuo. The product **21** was isolated after column chromatography (PE/EtOAc, gradient 1:1→1:3) as a white foam (R_f=0.33 with PE/EtOAc 1:2). Yield: 36% (over three steps based on **19**). ^{1}H NMR (600 MHz, $CDCl_3$, two rotamers): δ 1.44, 1.48 (9H, 2×s, C($CH_3)_3$), 1.81, 1.84 (3H, 2×s, CH_3-T), 2.33–2.46, 2.65–2.75 (2H, 2×m, CH_2S), 3.01–3.13, 3.43–4.55 (10H, 2×m, $CHCH_2O$, $CHCH_2N$, NCH_2CO, $NCOCH_2N$), 5.13 (0.5H, d, *J*=6.5 Hz, NH), 5.50 (0.5H, br s, NH), 6.81 (0.5H, s, H6), 6.85 (0.5H, d, *J*=1.0 Hz, H6), 7.18–7.75 (23H, m, H_{ar}), 9.00, 9.02 (1H, 2×br s, NH). ^{13}C NMR ($CDCl_3$, two rotamers): 12.23 (CH_3-T), 27.99 (C($CH_3)_3$), 33.41, 34.22 (CH_2S), 47.07, 47.17 ($CHCH_2O$), 47.25, 47.53, 49.49, 50.34, 50.47, 50 79 (CH_2), 49.43, 49.71 ($CHCH_2N$), 66.53, 66.67 ($CHCH_2N$), 67.03, 67.45 (C($Ph)_3$), 82.17, 83.36 (C($CH_3)_3$), 110.36, 110.46 (C5), 119.85, 119.92, 125.05, 125.19, 126.77, 126.95, 127.07, 127.61, 127.69, 127.94,

128.05, 129.47, 129.51 (CH_{ar}), 140.81, 141.12 (C6), 141.12, 141.23, 143.71, 144.07, 144.36, 144.48, 150.79, 150.85, 155.86, 155.93, 164.07, 164.12, 167.33, 167.86, 167.99, 168.30 (C_{ar}, CO).

LC–MS: 70–90% B, $t_R=13.9$ min. ES MS (found/calculated): 873.6/873.3 $(M+Na)^+$, HR-MS (found/calculated): 868.3699/868.3744 $(M+NH_4)^+$. $[\alpha]_D^{23}$ +17.6 (*c* 1 in $CHCl_3$). IR (cm^{-1}): 2360.7, 2351.2, 1670.2, 1222.8, 1149.5.

S-Isomer of **21**:

The synthesis was carried out in an identical manner to that described above. Spectroscopic data were identical. Yield: 32% (over three steps based on **19** (D)). $[\alpha]_D^{23}$ −17.0 (*c* 1 in $CHCl_3$).

4.1.11. (*R*) Fmoc-Cys(S*t*Bu)-thymine monomer *t*Bu ester (22). Iodine (12.07 g, 47.55 mmol) was dissolved in DCM (50 mL) and pyridine (5.38 mL, 66.57 mmol) was added. To this mixture was added a solution of compound **21** (2.7 g, 3.17 mmol) and 2-methyl-2-propanethiol (1.79 mL, 15.85 mmol) in DCM (50 mL). The reaction mixture was stirred at room temperature for 40 min and then the reaction was quenched by the addition of 0.5 M $Na_2S_2O_3$ (200 mL). The phases were separated and the organic layer was additionally washed with 0.5 M $Na_2S_2O_3$ (50 mL). The combined aqueous layers were extracted with EtOAc (3× 100 mL). The combined organic phases were dried ($MgSO_4$) and evaporated under reduced pressure. Compound **22** was purified by column chromatography (PE/EtOAc, gradient 1:1→1:3) to yield a white foam.

Yield: 86%; ^{1}H NMR (600 MHz, $CDCl_3$, two rotamers): δ 1.33, 1.35 (9H, 2×s, $SC(CH_3)_3$), 1.46, 1.50 (9H, 2×s, $OC(CH_3)_3$), 1.86, 1.88 (3H, 2×s, CH_3-T), 2.79–2.84, 2.95–3.04 (2H, 2×m, CH_2S), 3.48–4.69 (10H, m, $CHCH_2O$, $CHCH_2N$, NCH_2CO, $NCOCH_2N$), 5.64 (0.5H, d, J= 7.1 Hz, NH), 6.10 (0.5H, d, J=6.9 Hz, NH), 6.93 (0.5H, s, H6), 6.95 (0.5H, d, J=1.1 Hz, H6), 7.28–7.76 (8H, m, H_{ar}), 8.94 (1H, br s, NH). ^{13}C NMR ($CDCl_3$, two rotamers): δ 12.29 (CH_3-T), 27.99 ($OC(CH_3)_3$), 29.84 ($SC(CH_3)_3$), 41.76, 42.22 (CH_2S), 47.11, 47.19 ($CHCH_2O$), 47.58, 47.65, 48.32, 48.63, 49.73, 49.83, 50.64, 50.98 (CH_2, $SC(CH_3)_3$), 50.33, 50.59 ($CHCH_2N$), 66.77, 66.90 ($CHCH_2N$), 82.44, 83.60 ($OC(CH_3)_3$), 110.58, 110.71 (C5), 119.91, 119.97, 125.16, 125.26, 127.02, 127.66, 127.72 (CH_{ar}), 140.87, 141.18 (C6), 141.21, 141.26, 143.72, 143.78, 144.00, 150.90, 156.02, 164.05, 168.09, 168.23, 168.70 (C_{ar}, CO).

LC–MS: 50–90% B, $t_R=15.6$ min. ES MS (found/calculated): 697.3/697.3 $(M+H)^+$. HR-MS (found/calculated): 714.3022/714.2995 $(M+NH_4)^+$. $[\alpha]_D^{23}$ +9.6 (*c* 1 in $CHCl_3$). IR (cm^{-1}): 2358.7, 2340.4, 1666.8, 1222.5, 1149.4.

S-Isomer of **22**:

The synthesis was carried out in an identical manner to that described above. Spectroscopic data were identical. Yield: 71%. $[\alpha]_D^{23}$ −10.2 (*c* 1 in $CHCl_3$).

4.1.12. (*R*) Fmoc-Cys(S*t*Bu)-thymine monomer (23). Compound **22** (0.255 g, 0.366 mmol) was dissolved in DCM (0.5 mL) and TFA (4.5 mL) was added. The reaction mixture was stirred for 2 h and then the solvents were coevaporated under reduced pressure with toluene to give a white solid.

Yield: quantitative ^{1}H NMR (400 MHz, CD_3OD, two rotamers): δ 1.30, 1.33 (9H, 2×s, $SC(CH_3)_3$), 1.81 (3H, s, CH_3-T), 2.80–2.93 (2H, m, CH_2S), 3.30–3.36, 3.50–3.55, 3.63–3.71, 4.00–4.86 (10H, 4×m, $CHCH_2O$, $CHCH_2N$, NCH_2CO, $NCOCH_2N$), 7.09–7.81 (9H, m, H6, H_{ar}). ^{13}C NMR (CD_3OD, two rotamers): δ 12.24 (CH_3-T), 30.26 ($SC(CH_3)_3$), 43.19, 43.95 (CH_2S), 48.36, 50.73, 51.81, 52.06 (CH_2, $SC(CH_3)_3$), 48.47, 48.52 ($CHCH_2O$), 51.22, 51.47 ($CHCH_2N$), 67.74, 67.92 ($CHCH_2N$), 110.90, 110.99 (C5), 120.92, 126.30, 128.18, 128.78 (CH_{ar}), 142.60 (C_{ar}), 143.48 (C6), 145.29, 152.91, 158.39, 166.97, 169.94, 170.50, 172.01, 172.21 (C_{ar}, CO).

LC–MS: 20–90% B, $t_R=15.4$ min. ES MS (found/calculated): 641.2/641.2 $(M+H)^+$. HR-MS (found/calculated): 641.2073/641.2104 $(M+H)^+$. $[\alpha]_D^{23}$ −10.0 (*c* 1 in CH_3OH). IR (cm^{-1}): 2360.7, 2341.4, 1670.2, 1222.8, 1149.5.

4.1.13. Modified PNAs 25 and 26 and the reference PNA. The synthesis was performed on 10 μmol scale on an automated synthesizer (Applied Biosystems) using the Rink-Tentagel resin (loading capacity 0.22 mmol/g) and protocols supplied by the manufacturer. Deprotection/release from the resin was effected by suspension of the resin in a mixture of TFA/TIS/H_2O 90:5:5 (V, 5 mL) and filtered into Et_2O (40 mL). The precipitate was washed 1× with Et_2O, redissolved in H_2O–CH_3CN (3/1, 2 mL), and purified using the BIOCAD: 10–28% B over 5 CV.

Compound **25**: LC–MS: 5–35% B, $t_R=14.5$ min. Yield: 32% (estimated by A_{260} units). ES MS (found/calculated): $(M+2H)^{2+}$: 1578.0 (1577.6), $(M+3H)^{3+}$: 1052.6 (1052.1), $(M+4H)^{4+}$: 789.6 (798.3). MALDI-TOF MS (found/calculated) $(M+H)^+$: 3153.6 (3154.2).

PNA **26**: PNA **25** (1.59 mg, 0.5 μmol) was dissolved in 400 μL buffer (0.1 M Na_2HPO_4, 0.1 M TCEP) and the pH was adjusted to 6 with 1 M NaOH. After 4 h of agitation, 1% TFA/H_2O (400 μL) as well as 2 drops of neat TFA were added to dissolve all materials completely. Purification was performed on the BIOCAD VISION system.

Compound **26**: LC–MS: 5–35% B, $t_R=11.5$ min. Yield: 65% (estimated by A_{260} units). ES MS $(M+2H)^{2+}$: 1533.9 (1534.0), $(M+3H)^{3+}$: 1023.2 (1023.0), $(M+4H)^{4+}$: 767.6 (767.5), $(M+5H)^{5+}$: 614.2 (614.2). MALDI-TOF MS (found/calculated) $(M+H)^+$: 3068.2 (3067.0).

The reference PNA was synthesized as described for **25** (2 μmol scale, 9 mg of resin). Purification (10–25% B over 3 CV) gave the pure compound. LC–MS: 5–30% B, $t_R=11.9$ min, ES MS $(M+2H)^{2+}$: 1511.0 (1511.0), $(M+3H)^{3+}$: 1007.8 (1007.6), $(M+4H)^{4+}$: 756.9 (756.0). MALDI-TOF MS (found/calculated) $(M+H)^+$: 3021.5 (3020.9).

4.2. Chiral HPLC

Chiral HPLC experiments were executed using a Chiralcel OD column at 1 mL/min. A mixture of hexane–*i*-propyl-alcohol (92/8, V) containing 0.2% diethylamine was used as the eluent. The absorption was monitored at 254 nm. *R*-Enantiomer of **10**: $t_R = 10.4$ min, *S*-enantiomer of **10**: $t_R = 7.8$ min.

For compounds **21** a Chiralpak AD column together with the eluent isopropanol/hexane 1:1 (1 mL/min) was used: *R*-enantiomer of **21**: $t_R = 8.4$ min, *S*-enantiomer of **21**: $t_R = 15.5$ min.

4.3. Thermal denaturation studies

The melting temperature experiments were performed on a Perkin-Elmer lambda 20 spectrometer using a 1.0 mL cuvette with 1.0 μM of the two complementary strands in a phosphate buffered solution. Buffer A was prepared by mixing appropriate volumes of buffer 1 (200 mm sodium chloride, 20 mm NaH_2PO_4 and 0.2 mm EDTA) and buffer 2 (200 mm sodium chloride, 10 mm Na_2HPO_4 and 0.2 mm EDTA) until a pH value of 7.0 was obtained (using a pH-meter for determination of the pH value). The two complementary strands (dissolved in distilled H_2O) were added to 500 μL of buffer A. Distilled H_2O was added to a total volume of 1000 μL.

The samples were heated to 70 °C and cooled to 5 °C before initiating the experiment with a ramp of 1 °C/min. The melting temperature T_m was determined as the local maximum of the first derivative of the melting curve (A_{260} vs temperature).

References and notes

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