Solid-phase synthesis of novel peptide nucleic acids

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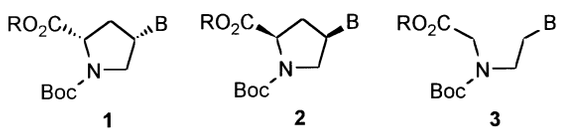
1 PERKIN

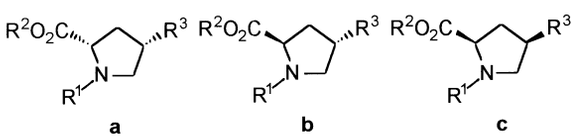
Novel peptide nucleic acids with configurationally and conformationally constrained glycylproline backbones have been synthesized by the solid-phase method from the Fmoc-protected dipeptide building blocks. Three decathymine peptide nucleic acids with different stereochemistry at the proline ring (*cis*-L, *trans*-D, *cis*-D) and mixed sequences in the *cis*-D series have been efficiently synthesized. In addition, oligothymine peptide nucleic acids with a conformationally flexible glycyl-*N*-ethylglycine backbone have also been synthesized from stepwise coupling of the Boc-protected monomer.

Introduction

The Boc-protected amino acids **1**–**3** where B is an appropriately protected or unprotected nucleobase have been synthesized, as have the Fmoc-protected dipeptides **4**–**8** for the synthesis of peptide nucleic acids (PNAs) with the configurationally and conformationally constrained glycylproline backbone **9** or the conformationally more flexible glycyl-*N*-ethylglycine backbone **10**.[1] We now report the synthesis of novel peptide nucleic acids from these building blocks.

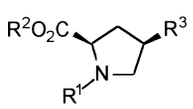


1 **2** **3**



a **b** **c**

4 R^1 = FmocGly, R^2 = H, R^3 = T
5 R^1 = FmocGly, R^2 = Pfp, R^3 = T

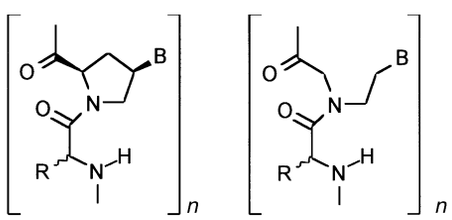


6 R^1 = FmocGly, R^2 = Pfp, R^3 = A^{Bz}
7 R^1 = FmocGly, R^2 = Pfp, R^3 = C^{Bz}
8 R^1 = FmocGly, R^2 = Pfp, R^3 = G^{Ibu}

Results and discussion

Since the Boc-protected monomer Boc-Eg(T)-OH (**3**; B = thymine; R = H) could be conveniently prepared on a multigram-scale, it was chosen as the precursor for the initial study of oligomer synthesis. An alternating sequence of a nucleobase-derivatised amino acid and another amino acid was required in order to provide a suitable spacing between adjacent nucleobases for a structural analogue of natural oligonucleotides.[1] Glycine was chosen as the spacer because it has neither a chiral centre to complicate peptide synthesis nor a side chain requiring protection.

The initial peptide targets were H-[Gly-Eg(T)]$_n$-Lys-OH (n = 6, 8, 10). Lysine was included at the C-terminus to prevent self-aggregation of the oligomers as suggested by Egholm and co-workers.[2] The presence of an additional positively charged hydrophilic side chain should also improve water solubility of the oligomers. All the couplings were carried out manually by a solid phase synthesis strategy[3] using the Boc/OBzl protection on phenylacetamidomethyl (PAM) resin.



9 **10**

Deprotection of the N-terminal Boc group was accomplished using 1:1 trifluoroacetic acid (TFA)–dichloromethane containing 0.2% dimethyl sulfide as a scavenger for the *tert*-butyl cation. Coupling with a primary amino terminus on the peptide chain was carried out using 2 mole equivalents of the Boc-protected amino acid in the presence of 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)[4]–diisopropylethylamine DIEA in dimethylformamide (DMF). The HBTU was replaced by bromotris(pyrrolidine)-phosphonium hexafluorophosphate (PyBrop), which is a superior reagent for coupling of hindered amino acids,[5] when the N-terminus of the resin-bound peptide is a secondary amino group. Each coupling was repeated twice followed by capping with Ac_2O–DIEA in DMF. The cycles were repeated until the 6-, 8- and 10-mers were obtained.

The peptide nucleic acids were released from the resin by trifluoromethanesulfonic acid (TFMSA). Following the standard cleavage procedure,[6] wash with diethyl ether and lyophilisation, crude products were obtained as solids. The peptide nucleic acids were purified by reversed phase HPLC on a C-18 column using acetonitrile–water containing 0.1% TFA gradient system. A small-scale purification of the hexamer with glycine as the spacer amino acid showed a single major peak accompanied by a few minor peaks which eluted before the desired product. The identity of the hexamer H-[Gly-Eg(T)]$_6$-Lys-OH was confirmed by electrospray mass spectrometry (ES-MS) which clearly showed the doubly charged molecular ion $[M + 2H]^{2+}$ (m/z 872.32). Mass spectra of higher oligomers suggested that they were contaminated by significant amounts of deletion sequences. Further HPLC purification on a gradient

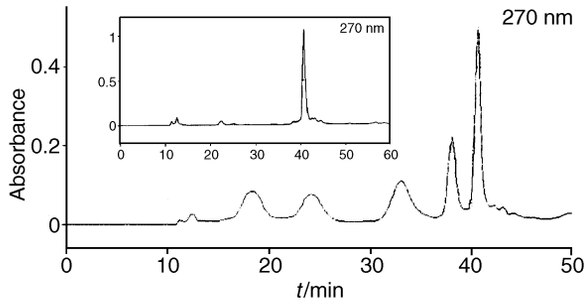


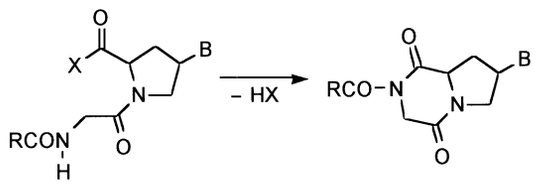
Fig. 1 Reversed-phase HPLC chromatogram of the crude octamer PNA H-[Gly-Eg(T)]$_8$-Lys-OH; Inset: HPLC chromatogram of the octamer after purification under identical conditions; HPLC conditions: μBondapak C-18 semipreparative column; isocratic 0.1% TFA in methanol (A) and 0.1% aq. TFA (B) (25:75 A:B) for 15 min, then linear gradient from 25:75–90:10 over a period of 45 min; flow rate 1.5 ml min^{-1}; detection wavelength 270 nm

system of water–methanol containing 0.1% TFA gave the pure hexamer, H-[Gly-Eg(T)]$_6$-Lys-OH, and octamer, H-[Gly-Eg(T)]$_8$-Lys-OH. All purified peptide nucleic acids appeared as fluffy solids after lyophilisation, and were readily soluble in water. Their aqueous solutions showed absorption maxima at 214 (peptide) and 268 nm (thymine) with minima at 230 nm. Electrospray mass spectra showed that they were free from truncated sequences. The materials obtained were sufficient for preliminary binding studies with natural oligonucleotides. A typical HPLC chromatogram of the octamer is shown in Fig. 1. The decamer proved too impure to be purified to homogeneity.

The poor yield of the peptide nucleic acids from stepwise coupling of protected amino acids (45% crude yield at 37% purity for the octamer) prompted us to explore the possibilities of increasing the efficiency of the synthesis by fragment coupling of dipeptides. The N-protecting group was also changed from Boc to Fmoc because of the milder Fmoc-deprotection conditions and the possibility of small-scale automated synthesis.

A model synthesis was first carried out manually on the *trans*-D-proline analogue. The target was a T_{10} peptide nucleic acid: H-[Gly-D-Pro(*trans*-4-T)]$_{10}$-Lys-NH_2. The lysine amide was included at the C-terminus for the reasons described earlier.[2] An acid-labile dimethoxybenzhydrylamine Novasyn-TGR resin was chosen as the solid support since cleavage with TFA leads directly to the peptide amide. The first lysine residue was introduced by coupling with Fmoc-Lys(Boc)-OPfp (OPfp = pentafluorophenyl ester) in the presence of *N*-hydroxybenzotriazole (HOBt). The peptide was synthesized from Fmoc-Gly-D-Pro(*trans*-4-T)-OH **4b**, in the presence of HBTU/DIEA according to the standard protocol for Fmoc-solid-phase synthesis.[7] The efficiency of the coupling reactions, which was followed quantitatively after deprotection of the Fmoc group by measuring the absorbance of the dibenzofulvene–piperidine adduct (ε_{264} 18 000 dm^3 mol^{-1} cm^{-1}) liberated during deprotection, was not as good as expected despite the use of a large excess of reagents and prolonged reaction times.

Much better coupling was obtained by first converting compound **4b** into the Pfp ester **5b** with dicyclohexylcarbodiimide (DCCI) and pentafluorophenol,[8] and then performing the coupling in the presence of HOBt. A possible reason for the low yield in the case of HBTU activation was that the activated monomer may have been lost by cyclisation to a diketopiperazine derivative (Scheme 1).[9] This is a very easy reaction for the active esters of protected or unprotected dipeptides which contain proline at the C-terminal, especially in the presence of a base; for example, Z-Gly-Pro-ONp (-ONp is 4-nitrophenyl ester) is known to cyclise spontaneously under basic conditions.[10] The Pfp ester is less reactive than the *O*-acylisourea or HOBt ester formed during HBTU activation and the coupling



Scheme 1 *Reagent:* base

does not require basic conditions, which probably explains the improved coupling yield. The preferred protocol for the peptide synthesis using Fmoc/OBu^t strategy and fragment condensation is summarised in Scheme 2.

Solid support: Novasyn TGR resin (dimethoxybenzhydrylamine-PEG) preloaded with Fmoc-Lys(Boc) (0.2 mmol g^{-1} loading)
Synthesis cycle:

Fmoc-group removal
20% piperidine in DMF, 15 min
↓
wash
DMF × 3
↓
coupling
4 mol equiv. Fmoc-dipeptide-OPfp/HOBt (0.1 M in DMF), 3 h
↓
wash
DMF × 3
↓
capping
5% Ac_2O in DMF, 15 min
↓
wash
DMF × 3

Final deprotection: 20% piperidine in DMF, 30 min
Cleavage: 95% TFA, 3 h; followed by precipitation with Et_2O and washing

Scheme 2

The decathymine peptide nucleic acids with different stereochemistry at proline (*trans*-D, *cis*-D and *cis*-L), *i.e.* H-[Gly-D-Pro(*trans*-4-T)]$_{10}$-Lys-NH_2, H-[Gly-D-Pro(*cis*-4-T)]$_{10}$-Lys-NH_2 and H-[Gly-L-Pro(*cis*-4-T)]$_{10}$-Lys-NH_2 were successfully prepared by successive coupling of the corresponding dipeptide Pfp esters according to the protocol shown in Scheme 2 on a 5 μmol scale. The syntheses were accomplished rapidly and much more efficiently than the stepwise coupling with Boc/OBzl (OBzl = benzyl ester) strategy employed earlier. Total amounts of activated dipeptides required for each 5 μmol synthesis of a decamer were approximately 150 mg. The peptide nucleic acids were released from the resin and purified according to the standard protocol. In each case, analytical HPLC of the crude products (C-18 column; a gradient system of water–acetonitrile containing 0.1% TFA) showed that they were 90–95% pure.

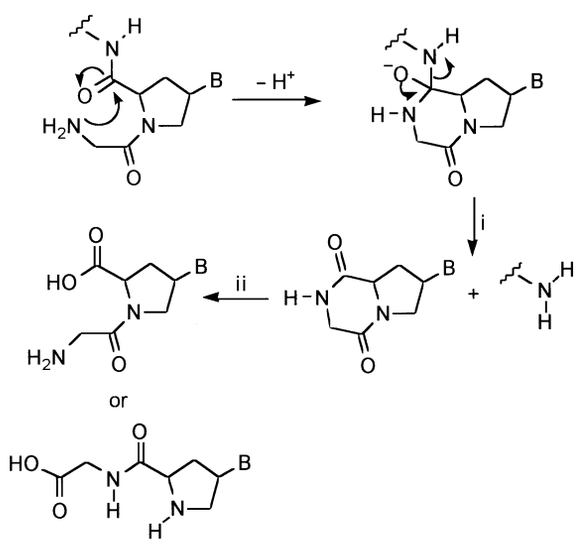
The peptides were purified by reversed phase HPLC and their identity confirmed by ES-MS (Table 1). Interestingly, these higher oligomers showed an ability to form adducts with alkali metal ions especially potassium in the mass spectrometer, as evidenced by the presence of mass peaks at M + 39*n*, where *n* is an integer, in addition to the expected molecular ion peak. In some cases, these potassium ion adducts appeared as the major peaks in the mass spectra. The total yield of the purified products was in the range of 40–55%. All the T_{10} peptide nucleic acids were sufficiently soluble in water for biological studies (>1 mg ml^{-1} at room temperature), although the *trans*-D analogue was considerably more soluble than the other two.

Next the incorporation of different nucleobases into the peptide nucleic acids was explored. The mixed adenine–thymine peptide nucleic acids of the *trans*-D and *cis*-L series were synthesized from the Pfp esters[1] without difficulty. However, attempts to remove the nucleobase protecting group (in this case, benzoyl) by treatment with aq. ammonia under various conditions resulted in degradation of the peptide as shown by

Table 1 ES-MS data of T_{10} PNAs. Experimental values are the average mass derived from various protonated species in the mass spectra

Peptide nucleic acid	M_r (Found)	M_r (Calc.)
H-[Gly-D-Pro(*trans*-4-T)]$_{10}$-Lys-NH_2	2927.53 ± 1.54	2927.88 [M]
	2968.29 ± 0.40	2965.97 [M − H + K]
	3005.07 ± 0.53	3004.26 [M − 2H + 2K]
H-[Gly-L-Pro(*cis*-4-T)]$_{10}$-Lys-NH_2	2929.02 ± 0.34	2927.88 [M]
	2967.27 ± 0.97	2965.97 [M − H + K]
	3005.88	3004.26 [M − 2H + 2K]
H-[Gly-D-Pro(*cis*-4-T)]$_{10}$-Lys-NH_2	2929.88 ± 2.81	2927.88 [M]
	2965.52 ± 0.36	2965.97 [M − H + K]
	3004.38 ± 1.39	3004.26 [M − 2H + 2K]
	3043.83 ± 0.66	3042.15 [M − 3H + 3K]

HPLC. It seemed unlikely that the degradation resulted from direct hydrolysis or ammonolysis of the peptide bond, since the Gly-Pro and Pro-Gly bonds are stable to hot aq. ammonia. HPLC and ES-MS analysis of the degradation products showed that they were the dipeptides Gly-Pro (or Pro-Gly) with the thymine moiety remaining attached (*m/z*: Calc. for M + H^+, 297). This suggested that the degradation was probably caused by intramolecular attack by the amino group of the N-terminal glycine on the amide carbonyl of the next residue to release the bicyclic diketopiperazine, which could undergo further hydrolytic ring opening under the deprotection conditions to form the corresponding dipeptide as observed in the mass spectrum (Scheme 3). The process would be repeated until the entire peptide chain was degraded. Such degradation reactions were frequently observed in peptides containing proline as the second residue from N-termini.[11] A similar degradation of PNA under basic conditions has also been reported.[12,13]



Scheme 3 *Reagents:* i, H^+; ii, water

Understanding the mechanism of degradation made it possible to avoid this serious side reaction by modifying the N-terminus of the peptide nucleic acid in a way that would diminish the nucleophilicity of the amino groups. It was therefore decided to find another protecting group which could be removed under conditions compatible with the peptide, preferably without introducing additional steps. The Boc group was used as it is labile under the conditions for peptide cleavage from the resin but stable under the conditions necessary to deprotect the nucleobases on the solid support. This protection-deprotection scheme was tested by synthesizing two mixed A–T sequences H-[Gly-L-Pro(*cis*-4-T)]$_2$-[Gly-L-Pro(*cis*-4-A)-Gly-L-Pro(*cis*-4-T)]$_2$-Lys-NH_2 and H-[Gly-L-Pro(*cis*-4-T)]$_6$-[Gly-L-Pro(*cis*-4-A)-Gly-L-Pro(*cis*-4-T)]$_2$-Lys-NH_2. The fully protected peptides were assembled on the solid support as usual and after the final removal of the *N*-Fmoc group, the free N-termini were capped with di-*tert*-butyl dicarbonate (Boc_2O) in the presence of DIEA in DMF. A qualitative ninhydrin test indicated that the coupling was essentially complete. After flushing of the reaction vessels with DMF, the resins were treated with 1:1 ethylenediamine–ethanol at room temperature overnight. This deprotection reagent has been used as a milder alternative to aq. ammonia for the base-labile methylphosphonate oligonucleotides.[14] The reagent was chosen here because the reaction could be carried out at room temperature and in the same vessel used for the peptide synthesis. Another advantage is that the resin swells better in this non-aqueous medium – swelling properties of the solid support are crucial for solid-phase reactions. The relatively non-volatile ethylenediamine and benzamide derivative from the cleavage reactions were easily removed by flushing the reaction vessels with DMF. Final cleavage and purification were carried out according to the standard method.[6,7] Reversed-phase HPLC analysis of the completely deprotected peptides showed clean single products in each case. The identity of the products was confirmed by mass spectrometry {H-[Gly-L-Pro(*cis*-4-T)]$_2$-[Gly-L-Pro(*cis*-4-A)-Gly-L-Pro(*cis*-4-T)]$_2$-Lys-NH_2: M_r, Calc. *m/z* 1832.84; Found: M^+, 1832.40 ± 0.10; H-[Gly-L-Pro(*cis*-4-T)]$_6$-[Gly-L-Pro(*cis*-4-A)-Gly-L-Pro(*cis*-4-T)]$_2$-Lys-NH_2: M_r, Calc. *m/z* 2945.92; Found: M^+, 2945.32 ± 0.14}.

The synthesis of PNAs incorporating all four natural nucleobases was undertaken next. The Pfp esters of protected adenine, cytosine and guanine Fmoc-dipeptides of the *cis*-D proline series (compounds **6**, **7** and **8**) had already been prepared.[1] The first model sequence synthesized was the tetramer H-Gly-D-Pro(*cis*-4-C)-Gly-D-Pro(*cis*-4-G)-Gly-D-Pro(*cis*-4-T)-Gly-D-Pro(*cis*-4-A)-Lys-NH_2. All the coupling was carried out under the conditions described for the oligothymine peptide nucleic acids. The coupling efficiency was monitored by measuring the absorbance of the dibenzofulvene–piperidine adduct from the deprotection step and showed that the guanine and cytosine could be introduced efficiently (>90% coupling yield, single coupling). The N-terminus of the resin bound peptide was then capped with the Boc group after removal of the last *N*-Fmoc group and then the resin was treated with conc. aq. ammonia–1,4-dioxane (1:1) at 55 °C overnight to remove the nucleobase protecting groups. Ethylenediamine was avoided in this instance because it had been shown to cause modification of the cytosine residue in oligonucleotides by displacement of the exocyclic amino group with the aminoethylamino group.[15] Final deprotection of the Boc group and cleavage from the solid support was carried out according to the standard protocol. Reversed-phase HPLC analysis revealed a single major product which was shown to be the desired product by ES-MS {H-Gly-D-Pro(*cis*-4-C)-Gly-D-Pro(*cis*-4-G)-Gly-D-Pro(*cis*-4-T)-Gly-D-Pro(*cis*-4-A)-Lys-NH_2: M_r, Calc. *m/z* 1276.54; Found: M^+, 1277.00 ± 0.07}. A decamer mixed-base PNA, H-Gly-D-Pro(*cis*-4-G)-Gly-D-Pro(*cis*-4-T)-Gly-D-Pro(*cis*-4-A)-Gly-D-Pro(*cis*-4-G)-Gly-D-Pro(*cis*-4-A)-Gly-D-Pro(*cis*-4-T)-Gly-D-Pro(*cis*-4-C)-Gly-D-Pro(*cis*-4-A)-Gly-D-Pro(cis-4-C)-Gly-D-Pro(*cis*-4-T)-Lys-NH_2, was also synthesized successfully by the standard protocol to give the product (M_r, Calc. *m/z* 2973.15;

Table 2 Some nucleopeptides prepared by Fmoc/OBu^t-fragment coupling strategy

PNA	t_R/min and HPLC conditions[a]	Purity of the crude product (%)[b]	Isolated yield (%)	Observed M_r (calc. M_r)[c]
H-[Gly-L-Pro(*cis*-4-T)]$_{10}$-Lys-NH_2	20.2 **A**	94	40	2927.53 ± 1.54 (2927.88)
H-[Gly-D-Pro(*trans*-4-T)]$_{10}$-Lys-NH_2	21.4 **A**	95	48	2929.02 ± 0.34 (2927.88)
H-[Gly-D-Pro(*cis*-4-T)]$_{10}$-Lys-NH_2	19.8 **A**	>90	55	2929.88 ± 2.81 (2927.88)
H-[Gly-D-Pro(*cis*-4-G)-Gly-D-Pro(*cis*-4-T)-Gly-D-Pro(*cis*-4-A)-Gly-D-Pro(*cis*-4-G)-Gly-D-Pro(*cis*-4-A)-Gly-D-Pro(*cis*-4-T)-Gly-D-Pro(*cis*-4-C)-Gly-D-Pro(*cis*-4-A)-Gly-D-Pro(*cis*-4-C)-Gly-D-Pro(*cis*-4-T)-Lys-NH_2	8.6 **B**	86	52	2974.80 ± 0.35 (2973.15)

[a] HPLC conditions: **A** 0.1% TFA in acetonitrile (A) and 0.1% TFA in water (B), A:B isocratic 10:90 for 5 min, then linear gradient from 10:90 to 90:10 during 35 min, flow rate 1.5 ml min^{-1}. **B** A:B 10:90 isocratic elution at flow rate 1.5 ml min^{-1}. [b] Calculated from analytical HPLC chromatogram at 270 (oligothymine) or 260 nm (mixed sequences). [c] The potassium adducts were not shown for clarity.

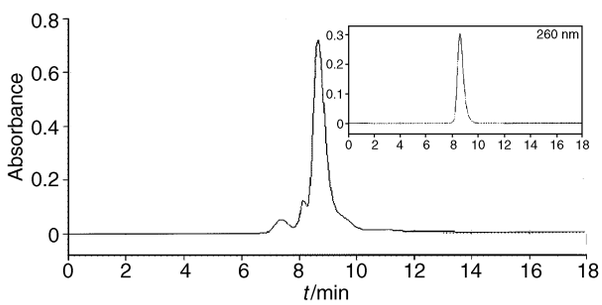


Fig. 2 Reversed-phase HPLC chromatogram of the crude decamer PNA H-Gly-D-Pro(*cis*-4-G)-Gly-D-Pro(*cis*-4-T)-Gly-D-Pro(*cis*-4-A)-Gly-D-Pro(*cis*-4-G)-Gly-D-Pro(*cis*-4-A)-Gly-D-Pro(*cis*-4-T)-Gly-D-Pro(*cis*-4-C)-Gly-D-Pro(*cis*-4-A)-Gly-D-Pro(*cis*-4-C)-Gly-D-Pro(*cis*-4-T)-Lys-NH_2; Inset: HPLC chromatogram of the purified PNA under identical conditions; HPLC conditions: μBondapak C-18 reversed-phase HPLC column; solvents: A = 0.1% TFA in acetonitrile, B = 0.1% aq. TFA isocratic (A:B 10:90); flow rate 1.5 ml min^{-1}; detection wavelength 260 nm

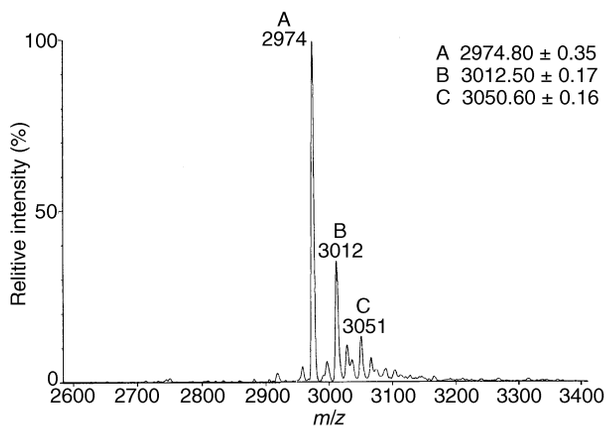


Fig. 3 ES-mass spectrum of the purified PNA decamer H-Gly-D-Pro(*cis*-4-G)-Gly-D-Pro(*cis*-4-T)-Gly-D-Pro(*cis*-4-A)-Gly-D-Pro(*cis*-4-G)-Gly-D-Pro(*cis*-4-A)-Gly-D-Pro(*cis*-4-T)-Gly-D-Pro(*cis*-4-C)-Gly-D-Pro(*cis*-4-A)-Gly-D-Pro(*cis*-4-C)-Gly-D-Pro(*cis*-4-T)-Lys-NH_2. The potassium adducts [M_r = 3012 (M + K^+ − H^+) and 3051 (M + 2K^+ − 2H^+)] were also observed in addition to the product (M_r = 2974).

Found: [M + H]$^+$ 2974.80 ± 0.35) in good yield and purity after purification by reversed-phase HPLC (Table 2). The reversed-phase HPLC chromatogram and ES-MS analysis of the decamer are shown in Fig. 2 and Fig. 3.

A report on the hybridisation of these novel PNAs with oligonucleotides and nucleic acids will be described elsewhere.

Experimental

The solid supports, PAM resin initially loaded with Boc-Lys-(2-ClZ)-OH (0.42 mmol g^{-1}) (Boc/OBzl strategy) and Novasyn™ TGR resin (~ 0.23 mmol free NH_2 group per g) (Fmoc/OBu^t strategy), were obtained from Calbiochem-Novabiochem Ltd. The protected amino acids and derivatives and the coupling reagents (HBTU, PyBrop) were also obtained from the same source. The protected nucleo-amino acids, dipeptides and Pfp esters were prepared as described previously.[1] TFA (98%) was obtained from Avocado Research Chemicals Ltd. All other reagents were obtained at highest purity grade available either from Aldrich Chemical Company Ltd. or Lancaster Synthesis Ltd. and were used as received. Reagents for the Kaiser test were prepared according to the literature.[16]

DMF was peptide synthesis grade obtained from Rathburn Chemicals Ltd. and used without further purification. All other solvents used for the synthesis and purification were HPLC-grade solvents obtained from Rathburn. Deionised water was obtained from an Elga Maxima Ultra-Pure water purification system. The solid-phase syntheses were conducted manually on a DuPont RaMPS™ Multiple Peptide Synthesis Processor or in a syringe as discussed in detail below.

Samples for reversed-phase HPLC analysis were dissolved in a suitable aqueous solvent and filtered through a Teflon filter (0.47 μ pore size, Anachem Ltd.). HPLC was performed on a Waters 990+ system with a diode array detector. A Waters μBondapak C-18 semi-preparative reversed-phase HPLC column (0.78 × 30 cm, P/N 84176) was used for both analysis and preparative purposes. Peak monitoring and data analysis were performed on Waters 990 software running on an NEC IBM-PC/AT compatible computer with 80286/80287 microprocessors. The samples were recovered from HPLC fractions by freeze drying on a VirTis Freezemobile 5SL freeze drier. ES-mass spectra of the peptide nucleic acids were recorded on a VG Biotech-BioQ or VG Biotech Platform mass spectrometer.

Solid-phase peptide synthesis of peptide nucleic acids with the glycyl-*N*-ethylglycine backbone using the Boc/OBzl-stepwise coupling strategy

All syntheses were conducted on a 50 μmol scale on the RaMPS synthesizer. Three 50 μmol portions of the resin (119.0 mg each) were weighed into 3 separate peptide synthesis cartridges. Another 50 mg of the resin was weighed into another cartridge as a control.

The synthesis cycle was started by deprotection of the N-terminal Boc-protecting group using a 1:1 mixture of TFA–dichloromethane containing 0.2% dimethyl sulfide. The deprotection mixture (2 ml) was added to each cartridge, except the control cartridge, and drained quickly. Another 2 ml of the deprotection mixture was added and the cartridges were shaken

at room temperature. After 20 min, the cartridges were drained and the contents washed well with methanol. The resin beads were reswollen in dichloromethane and drained, then washed with methanol again. The wash procedure was repeated 3 times to remove as much of the excess of reagents as possible. The beads were shrunken with methanol and washed with 10% DIEA in dichloromethane.

After alternate washes with methanol and dichloromethane as described, DMF or 20% trifluoroethanol in DMF was added to each cartridge and drained. Appropriate protected amino acids (2.0 mol equiv.) were separately dissolved in 1.5 ml of DMF in small vials. An appropriate coupling reagent [HBTU (2.0 mol equiv.), or PyBrop (2.0 mol equiv.)] was added and the solutions were transferred to the cartridges followed by 50 µl (0.28 mmol, 5.7 mol equiv.) of DIEA. The cartridges were capped and shaken at room temperature. After 2 h, the cartridges were drained and the beads were washed well with methanol and dichloromethane. The coupling procedure was repeated again for each residue and the beads were washed with methanol and air dried.

Kaiser tests for successful coupling reactions were performed as described in the literature.[12] This usually resulted in negative results (*i.e.* efficient coupling). If the coupling was incomplete, as indicated by coloured beads or solutions, a capping procedure was conducted as follows: The beads were swollen in DMF (2.0 ml) and shaken with Ac_2O (200 µl, 2.1 mmol) and DIEA (100 µl, 0.57 mmol) for 1 h, washed well and drained. The next cycle was then started with the deprotection step as above. The synthesis was interrupted when the hexamers were obtained. The beads in each cartridge were split into three equal parts. Two parts were returned to the cartridge and the synthesis was continued. The hexamers were cleaved from the rest of the resin as described below. The same method was applied when the synthesis had reached the octamer stage. The synthesis was stopped after the decamers were obtained. The peptides were cleaved from the resin by treatment with TFMSA followed by precipitation with diethyl ether and were purified by reversed-phase HPLC.

Solid-phase synthesis of peptide nucleic acids with configurationally and conformationally constrained backbone using Fmoc/OBu^t-fragment coupling strategy

In a polyethylene syringe (1 ml) equipped with a removable stainless steel needle fitted with a glass wool plug at the junction was placed the Novasyn TGR resin [preloaded with Fmoc-Lys(Boc)-OH 0.23 mmol g^{-1}; 10–25 mg, ~2.5–5 µmol]. The needle was then inserted through a rubber septum fitted to a Büchner flask. Washing was carried out by adding solvent from the top of the syringe with the plunger removed and sucking into the receiver flask by a water aspirator. For the deprotection, coupling and capping stages, the plunger was reattached and the reagent was taken up through the needle. Deprotection of the Fmoc group was accomplished by treatment of the Fmoc-peptide resin with freshly prepared 20% piperidine in DMF (1.0 ml on a 5 µmol scale synthesis) for 20 min with occasional agitation. After the specified period of time the reagent was ejected by depressing the plunger and washing carried out as described above. The solution from the deprotection stage containing the dibenzofulvene–piperidine adduct was collected and the optical absorbance at 264 nm was measured to assess the efficiency of the previous coupling stage. The resin had to be washed exhaustively to ensure complete removal of the piperidine. The coupling was carried out similarly using typically 4 mole equivalents of the Fmoc-dipeptide Pfp ester and 4 mole equivalents of $HOBt\cdot H_2O$ in DMF with a final concentration of the Pfp ester at ~ 0.1 M. Generally the coupling was completed within 3 h and no second coupling was required. In cases where incomplete coupling was suspected, the peptide resin was treated with 5% Ac_2O in DMF (1 ml for a 5 µmol scale synthesis) for 30 min at room temperature to prevent the formation of deletion sequences. The acetylating mixture was ejected and the reaction vessel was flushed with DMF 3 times before the deprotection–coupling–capping steps were repeated until the last peptide fragment had been added. The N-terminal Fmoc group was removed by 20% piperidine in DMF and the resin was washed with DMF. If the sequence contained only thymine or if the exocyclic amino protecting groups were to be retained, the cleavage of the peptide nucleic acid from the resin with TFA was performed directly. However, when a fully deprotected peptide nucleic acid containing adenine, cytosine and/or guanine was required, the resin-bound peptide was capped with a Boc group prior to the cleavage as described below.

Deprotection of exocyclic amino protecting groups on nucleobases (A, C and/or G containing sequences only) *via* a temporary Boc-protection

The resin-bound peptide containing a free amino terminus (5 µmol scale synthesis) was treated with a solution of Boc_2O (50 µl, 22 µmol) and DIEA (35 µl, 20 µmol) in DMF (150 µl) at room temperature. Kaiser testing indicated complete reaction after 3 h. The resin was washed several times with DMF and then the exocyclic amino protecting groups were removed by treatment with a 1:1 mixture of ethylenediamine and 95% ethanol (200 µl) at room temperature overnight. For cytosine-containing sequences, the PNAs were treated with 1:1 conc. aq. ammonia–1,4-dioxane at 55 °C for 16 h instead of ethylenediamine–ethanol in order to avoid the transamination reaction at cytosine residues. The resin-bound deprotected peptide nucleic acid from either method was washed successively with DMF and methanol, and air dried.

Cleavage of peptide nucleic acids from the solid support and purification

The peptide nucleic acid on the solid support, either with the free amino terminus or carrying the Boc group, was washed with methanol and air dried. It was then treated with TFA containing 5% water (~1 ml for a 5 µmol synthesis) at room temperature for 2.5 h with occasional agitation. After the specified period of time, the cleavage solution was diluted with diethyl ether (ten times the volume) and kept at −20 °C overnight. The suspension was then centrifuged at 13 000 rpm for 5 min. After decantation of the supernatant, the crude PNA was washed with diethyl ether and the centrifugation–wash process was repeated 4–5 times. Finally the crude PNA was air dried and dissolved in 10% aq. acetonitrile containing 0.1% TFA. The crude solution was filtered, and analysed, or purified by reversed-phase HPLC. Sample elution was carried out using a gradient of water–acetonitrile containing 0.1% TFA.

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