Synthesis and Properties of Novel Peptide Nucleic Acids with Hydrophilic Serine Spacers

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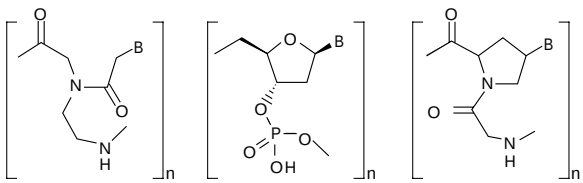
ABSTRACT Novel conformationally constrained peptide nucleic acids (PNA) with alternate thymine-modified proline and serine residues were synthesized with the aim of improving the solubility of the resulting PNA in aqueous media. The PNAs were synthesized from the corresponding activated Fmoc-protected dipeptide monomers employing solid phase methodology and were characterized by MALDI-TOF mass spectrometry. Binding to their complementary oligodeoxyribonucleotide of T_{10} PNA homo-oligomer carrying either D- or L-serine spacer was studied by UV spectrophotometry and gel electrophoresis.

KEYWORDS: peptide, nucleic acid, oligonucleotide, hydrophilic.

INTRODUCTION

Peptide nucleic acid (PNA) (1) is a class of oligonucleotide analogue in which the sugar phosphate backbone of DNA (2) is replaced with an isostructural aminoethylglycine pseudopeptide unit.[1-8] PNA has attracted much attention in the field of antisense research[9] due to its unique combination of properties not found in natural oligonucleotides, including high affinity and specificity towards complementary RNA and DNA, and stability towards nucleases and proteases. According to molecular modelling, it was suggested that single stranded PNA might be preorganized by intramolecular hydrogen bonding thus facilitating binding to its complementary nucleic acid.[10,11] The three dimensional structure of hybrids between PNA and oligonucleotides as well as PNA-DNA and PNA-RNA hybrids have been studied in details by X-ray crystallography[12] and NMR spectroscopy.[13,14] The strikingly high affinity of PNA towards complementary nucleic acid was ascribed to the lack of electrostatic repulsion between the uncharged PNA backbone and the negatively charged sugar phosphate backbone of the nucleic acid in addition to the usual base-base pairing and stacking. In spite of a number of valuable properties, PNA (1) suffers from a tendency to self-aggregrate, has poor water solubility and an inability to penetrate cell membranes, therefore a number of modifications have been made in an attempt to circumvent these difficulties without compromising specificity and binding affinity. Recent examples of such modifications include: introduction of substituents to the CH_2 group in (1) to create chirality,[15,16] introduction of hydrophilic groups[17] or positively-charged groups[18,19] onto the PNA backbone, or restricting the movement of the backbone or the side chain of PNA, *e.g.* by replacing the amide bond with a double bond,[20,21] or by making the backbone or side-chain part of a carbo- or hetero-cycle.[22-25]

So far these modifications have resulted in varying degrees of success. The resulting modified PNA seldom show comparable or better binding affinity to complementary oligonucleotides than PNA (1).[17,19] We proposed that replacement of the flexible PNA backbone with a glycylproline dipeptide unit would restrict the conformation of the resulting PNA and lead to better binding to complementary nucleic acids for entropic reasons. Therefore chiral PNAs containing the nucleobase-modified glycylproline backbone (3) were synthesized and their binding properties studied.[26-28] Indeed, oligomers bearing *cis*-D (3, B = Thymine; n=10; stereochemistry = 2*R*,4*R*) and *cis*-L (3, B = Thymine; n=10; stereochemistry = 2*S*,4*S*) stereochemistries at proline were shown to hybridize with poly(dA) with high affinity (T_m = 69°C and 70°C respectively).[29] Although preliminary experiments suggested that hybridization between *cis*-D (3) and $(dA)_{10}$ is probably by Watson-Crick base-pairing, structural studies of the interaction by NMR spectroscopy were precluded by the poor solubility in aqueous medium



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of the proline-containing PNA-oligonucleotide hybrid. In order to overcome this difficulty, we have replaced glycine by serine in the PNA analogues reported here. As there are two enantiomers of serine and the stereochemistry of the spacer amino acid may have a significant effect on the binding strength to complementary oligonucleotides, both enantiomers were included in this study.

Materials and Methods

General

Melting points were recorded on a Kofler block apparatus. Specific rotations were measured on a Perkin-Elmer 241 polarimeter (Dyson Perrins Laboratory, Oxford). IR spectra were recorded on a Perkin-Elmer 1750 (Dyson Perrins Laboratory, Oxford) or a Nicolet Impact 410 Fourier Transform Infrared spectrometer (Chulalongkorn University, Bangkok). Elemental analyses were performed at Chulalongkorn Research Equipment Centre on a Perkin-Elmer CHN analyser model PE2400 series II.

Routine ^{1}H and ^{13}C NMR spectra were recorded on a Bruker ACF 200 (Chulalongkorn University, Bangkok) operating at 200 MHz (^{1}H) and 50.28 MHz (^{13}C). ^{13}C spectra were recorded in broad band decoupled mode and the chemical shift assignment was assisted by a DEPT experiment. High field NMR experiments were performed on a Bruker DRX400 (400 MHz) (National Science and Technology Development Agency, Bangkok) or a JEOL-JNM500 (Chulalongkorn Research Equipment Centre, Bangkok) NMR spectrometer. ^{1}H and ^{13}C chemical shifts are quoted in ppm relative to tetramethylsilane and were internally referenced to the residual protonated solvent signal. Positive Chemical Ionization Mass Spectrometry was carried out in APCI mode in 1:1 methanol:dichloromethane as a carrier solvent (Dyson Perrins Laboratory, Oxford) and MALDI-TOF mass spectra were recorded on a Bruker Biflex mass spectrometer (Institute for Genetic Engineering and Biotechnology, Chulalongkorn University, Bangkok).

The solid support for peptide synthesis, Novasyn™ TGR resin (~0.20-0.25 mmol free NH_2 group/g) and protected amino acids [Fmoc-L-Ser(^{t}Bu)-OH, Fmoc-D-Ser(^{t}Bu)-OH, Fmoc-Gly-OPfp, Fmoc-Lys(Boc)-OPfp] were obtained from Calbiochem-Novabiochem Ltd. Other reagents and solvents were obtained from standard suppliers and used without further purification.

HPLC was performed on a Waters 990+ system with a diode array detector. An Alltima C-18 reverse phase HPLC column (4.6x150 mm, C/N 09-81385), 3 µ particle size was used for both analysis and semi-preparative purposes. The samples were recovered from HPLC fractions by freeze drying.

Synthesis of Monomers

*N-(*N-*Fluoren-9-ylmethoxycarbonyl*-O-*t-butyl-L-seryl)*-cis-*4-(*N^{3}-*benzoylthymin-1-yl)-D-proline diphenylmethyl ester (7a)*

N-tert-Butoxycarbonyl-*cis*-4-(*N*^{3}-benzoylthymin-1-yl)-D-proline diphenylmethyl ester [*N*-Boc-D-Pro(*cis*-T^{Bz})-ODpm] (**6**)[27] (350 mg, 0.58 mmol) was dissolved in acetonitrile (5mL). *p*-Toluenesulfonic acid monohydrate (552 mg, 2.9 mmol) was added and the solution was stirred at room temperature for 1.5 h, after which tlc indicated complete deprotection of the *N*-Boc group. Diisopropylethylamine (515 mL, excess) and *N*,*N*'-dimethylformamide (DMF) (5 mL) were added with stirring under nitrogen. In a separate reaction vessel, a mixture of Fmoc-L-Ser(^{t}Bu)-OH (268 mg, 0.70 mmol), hydroxybenzotriazole hydrate (HOBt.H_2O) (118 mg, 0.77 mmol) and dicyclohexylcarbodiimide (DCCI) (160 mg, 0.78 mmol) in DMF (2 mL) was stirred at room temperature. After 2 hours, a white precipitate of dicyclohexylurea was removed by filtration and the filtrate transferred to the first reaction vessel. The reaction mixture was stirred at room temperature for a further 3 h, diluted with dichloromethane (50 mL), and washed with saturated aqueous $NaHCO_3$ and water. The organic phase was dried ($MgSO_4$) and the solvent removed under reduced pressure to give the crude product as an oil which was purified by column chromatography (SiO_2; dichloromethane : acetone 20:1). The product (R_f = 0.43) was obtained as a white solid (455 mg, 89 %), m.p. 102-105°C, (Found C, 71.8 ; H, 5.5; N, 6.3 %; $C_{52}H_{50}N_4O_9$ requires C, 71.4; H, 5.8; N, 6.4 %), δ_H (500 MHz; $CDCl_3$) 1.14 [9H, s, ^{t}Bu C$\underline{H}_3$], 1.81 [3H, s, thymine C$\underline{H}_3$], 2.05 and 2.52 [2H, 2xm, C$\underline{H}_2$(3')], 3.45 and 3.65 [2xH, 2xm, Ser C$\underline{H}_2$], 3.80 [1H, m, 1xC$\underline{H}_2$(5')], 4.28-4.42 [3H, m, Fmoc aliphatic C$\underline{H}$, C$\underline{H}_2$], 4.50 [1H, m, 1xC$\underline{H}_2$(5')], 4.68 [2H, m, Ser C$_\alpha\underline{H}$], 4.75 [2H, m, C$\underline{H}$(2')], 5.26 [C$\underline{H}$(4')], 5.62 [1H, d *J* = 8.2 Hz, peptide N$\underline{H}$], 6.88 [1H, s, C$\underline{H}$Ph$_2$], 7.12 [1H, s,

C(6)H], 7.22-7.66 [m, phenyl, Fmoc and benzoyl aromatic CH], 7.90-7.94 and 8.04-8.08 [2x2H, 2xd, Fmoc aromatic CH]; δ_C (125.65 MHz; $CDCl_3$) 12.4 [thymine CH_3], 27.3 [^{t}Bu CH_3], 33.3 and 33.9 [CH_2(3')], 47.0 [Fmoc aliphatic CH], 49.5 [CH_2(5')], 52.4 and 52.5 [Ser $C_\alpha H$ and CH(4')], 57.5 [CH(2')], 63.3 [Ser CH_2], 67.2 [Fmoc CH_2], 73.9 [^{t}Bu C], 78.5 [$CHPh_2$], 112.0 [C(5)], 120.0 [Fmoc aromatic CH], 125.1-130.4 [aromatic CH], 135.0 and 135.3 [C(6)H and benzoyl *p*-CH], 139.0 and 139.4 [aromatic C rotamers], 141.2 143.6 and 143.8 [Fmoc aromatic C], 149.7 [C(2)], 155.7 [Fmoc CO], 162.2 [C(4)], 168.6 [benzoyl CO], 170.1 [peptide CO], 170.4 [ester CO]; *m/z* (APCI+) 931 ($M+C_4H_8^+$, 90 %), 876 ($M+H^+$, 88), 654, 587, 550, 503, 409, 165, 105 ($PhCO^+$, 100); ν_{max} (KBr)/cm^{-1} 1750s, 1700s and 1660s (C=O); $[\alpha]_D^{26}$ +12.3 (c=1.02, $CHCl_3$).

N-*(*N-*Fluoren-9-ylmethoxycarbonyl*-O-*t-butyl-D-seryl)*-cis-*4-(*N^3-*benzoylthymin-1-yl)-D-proline diphenylmethyl ester (7b)*

The titled compound was obtained in analogous manner to the isomer (7a) described above as a white foam [78%, starting from 1.08 mmol of Boc-protected proline derivative and 1.20 mmol of Fmoc-D-Ser(^{t}Bu)-OH] after column chromatography: m.p. 118-120°C, (Found C, 71.3 ; H, 6.0; N, 6.4 %; $C_{52}H_{50}N_4O_9$ requires C, 71.4; H, 5.8; N, 6.4 %), δ_H (500 MHz; $CDCl_3$) 1.18 and 1.21 [9H, 2xs, ^{t}Bu CH_3 rotamers], 1.70 and 1.85 [3H, 2xs, thymine CH_3 rotamers], 2.00-2.17 and 2.55-2.85 [2H, 2xm, CH_2(3')], 3.37-3.75 [2H, m, Ser CH_2], 4.00-4.15 [1H, m, 1xCH_2(5')], 4.15-4.40 [3H, m, 1xCH_2(5') and Fmoc aliphatic CH, CH_2], 4.63-4.84 [2H, m, CH(2') and Ser $C_\alpha H$], 5.21-5.40 [CH(4')], 5.80-5.84 [1H, m, peptide NH], 6.96 [1H, m, $CHPh_2$ rotamers], 7.23 [1H, s, C(6)H], 7.25-7.97 [m, phenyl, Fmoc and benzoyl aromatic CH]; δ_C (125.65 MHz; $CDCl_3$) 12.4 and 12.5 [thymine CH_3 rotamers], 27.2 [^{t}Bu CH_3], 33.4 and 33.9 [CH_2(3') rotamers], 47.0 [Fmoc aliphatic CH], 49.0 and 50.3 [CH_2(5') rotamers], 52.0 and 52.3 [Ser $C_\alpha H$ rotamers], 53.0 and 53.3 [CH(4') rotamers], 57.6 and 57.8 [CH(2') rotamers], 63.2 and 64.0 [Ser CH_2 rotamers], 67.1 [Fmoc CH_2], 73.9 and 74.0 [^{t}Bu C rotamers], 78.4 and 79.5 [$CHPh_2$ rotamers], 111.9 [C(5)], 120.2 [Fmoc aromatic CH], 125.4-131.7 [aromatic CH], 135.4 and 136.4 [C(6)H and benzoyl *p*-CH], 139.1 and 139.5 [aromatic C rotamers], 143.6 and 143.8 [Fmoc aromatic C], 149.7 [C(2)], 155.8 [Fmoc CO], 162.2 [C(4)], 168.6 [benzoyl CO], 170.3 [peptide CO], 170.4 [ester CO]; *m/z* (APCI+) 930 ($M+C_4H_8^+$, 26 %), 876 ($M+H^+$, 38), 708 (32), 653 (75), 587, 550, 503, 167 (Ph_2CH^+, 100); ν_{max} (KBr)/cm^{-1} 1750s, 1700s and 1660s (C=O); $[\alpha]_D^{26}$ +24.3 (c=1.05, $CHCl_3$).

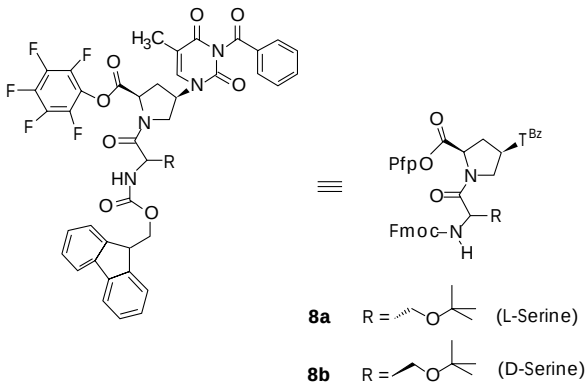
General procedure for C-terminal deprotection of N-*(*N-*Fluoren-9-ylmethoxycarbonyl*-O-*t-butylseryl)*-cis-*4-(*N^3-*benzoylthymin-1-yl)-D-proline diphenylmethyl ester.*

The protected dipeptide was dissolved in methanol containing 10 % Pd/C (*ca* 30 % wt of the protected dipeptide) and stirred under atmosphere of hydrogen at room temperature for 1 hr, after which tlc indicated complete cleavage of the Dpm ester. The reaction mixture was then filtered through Celite to remove the catalyst and the solvent was evaporated to give the product as a white solid. The crude product was used for the next step without further purification.

General procedure for synthesis of N-*(*N-*Fluoren-9-ylmethoxycarbonyl*-O-*t-butylseryl)*-cis-*4-(* N^3-*benzoylthymin-1-yl)proline pentafluorophenyl ester*

A mixture of the *N*-Fmoc-dipeptide free acid, pentafluorophenol (1.1 eq) and DCCI (1.1 eq) in dichloromethane was stirred at room temperature for 2-3 hr. The precipitated dicyclohexylurea was filtered off, the solvent removed under reduced pressure and the residue purified by column chromatography (SiO_2, dichloromethane:acetone or EtOAc).

N-(N-Fluoren-9-ylmethoxycarbonyl-O-t-butyl-L-seryl)-cis-4-(N^3*-benzoylthymin -1-yl)-D-proline pentafluorophenyl ester (8a)* was obtained as a white solid (50 %, starting from 1.46 mmol of the protected dipeptide 7a) m.p. 112-113°C, (Found C, 61.7 ; H, 4.8; N, 6.5 %; $C_{45}H_{39}F_5N_4O_9$ requires C, 61.8; H, 4.5; N, 6.4 %), δ_H (500 MHz; $CDCl_3$) 1.16 [9H, s, ^{t}Bu CH_3], 1.97 [3H, s, thymine CH_3], 2.33-2.42 and 2.90-2.99 [2H, 2xm, CH_2(3')], 3.47-3.54 and 3.64-3.67 [2H, 2xm, Ser CH_2], 3.88-4.03 [1H, m, 1xCH_2(5')], 4.17-4.23 [1H, m, Fmoc aliphatic CH], 4.32-4.41 [2H, m, Fmoc aliphatic CH_2], 4.46-4.54 [1H, m, 1xCH_2(5')], 4.68-4.76 and 4.80-4.86 [2H, 2xm, CH(2') and Ser $C_\alpha H$], 5.26-5.34 [CH(4')], 5.57 - 5.62 [1H, d *J* = 8.2 Hz, peptide NH], 7.27-7.32 [1H, s, CH(6)], 7.34-7.58 [m, CH(6), phenyl, Fmoc and benzoyl aromatic CH], 7.63-7.68 [2H, m, Bz *o*-CH], 7.73-7.78 and 7.89-7.93 [2x2H, 2xd, Fmoc aromatic CH]; δ_C (125.65 MHz; $CDCl_3$) 12.3 [thymine CH_3], 27.3 [^{t}Bu CH_3], 33.7 [CH_2(3')], 47.0 [Fmoc aliphatic CH], 49.1 [CH_2(5')], 52.5 and 53.2 [Ser $C_\alpha H$ and CH(4')], 56.8 [CH(2')], 63.0 [Ser CH_2], 67.4 [Fmoc CH_2], 74.0 [^{t}Bu C], 112.1 [C(5)], 120.0 [Fmoc aromatic CH], 125.0-130.4 [aromatic CH], 135.1 and 135.4 [C(6)H and benzoyl *p*-CH], 141.2 and 141.3 [pentafluorophenyl], 143.4 and 143.7 [Fmoc aromatic C], 149.8 [C(2)], 156.0 and 157.2[Fmoc CO rotamers], 162.3 [C(4)], 167.1 [benzoyl CO], 168.5 [peptide CO], 171.1 [ester CO]; ν_{max} (KBr)/cm^{-1} 1801s, 1752s, 1702s and 1660s (C=O).



N-(N-Fluoren-9-ylmethoxycarbonyl-O-t-butyl-D-seryl)-cis-4-(benzoylthymin-1-yl)-D-proline pentafluorophenyl ester (8b) was obtained as a white solid (20 %, starting from 1.46 mmol of the protected dipeptide 7b) m.p. 109-110°C, (Found C, 61.8 ; H, 4.6; N, 6.3 %; $C_{45}H_{39}F_5N_4O_9$ requires C, 61.8; H, 4.5; N, 6.4 %), δ_H (500 MHz; $CDCl_3$) 1.17 [9H, s, ^{t}Bu C$\underline{H}_3$], 1.96 [3H, s, thymine C$\underline{H}_3$], 2.34-2.42 and 2.91-2.99 [2H, 2xm, C$\underline{H}_2$(3')], 3.51-3.57 and 3.61-3.66 [2H, 2xm, Ser C$\underline{H}_2$], 4.06-4.12 [1H, m, C$\underline{H}_2$(5')], 4.18-4.21 [1H, m, Fmoc aliphatic CH], 4.31-4.35 [1H, m, C$\underline{H}_2$(5')], 4.36-4.38 [1H, m, Fmoc aliphatic CH], 4.60-4.69 [1H, m, Ser C$_\alpha\underline{H}$], 4.85-4.90 [1H, m, C$\underline{H}$(2')], 5.28-5.35 [C$\underline{H}$(4')], 5.64 - 5.70 [1H, d *J* = 8.2 Hz, peptide N$\underline{H}$], 7.22 [1H, s, C(6)$\underline{H}$], 7.31-7.60 [m, phenyl, Fmoc and benzoyl aromatic CH], 7.55-7.59 [2H, m, Bz *o*-C$\underline{H}$], 7.73-7.78 and 7.89-7.94 [2x2H, 2xd, Fmoc aromatic C$\underline{H}$]; δ_C (125.65 MHz; $CDCl_3$) 12.6 [thymine C$\underline{H}_3$], 27.2 [^{t}Bu C$\underline{H}_3$], 33.1 and 33.7 [C$\underline{H}_2$(3') rotamers], 47.0 [Fmoc aliphatic C$\underline{H}$], 49.7 [C$\underline{H}_2$(5')], 52.8 and 53.9 [Ser C$_\alpha$H and C$\underline{H}$(4')], 57.2 [C$\underline{H}$(2')], 63.2 [Ser C$\underline{H}_2$], 67.3 [Fmoc C$\underline{H}_2$], 74.0 [^{t}Bu C$\underline{}$], 112.3 [C$\underline{}$(5)], 120.0 [Fmoc aromatic C$\underline{H}$], 125.0-130.4 [aromatic C$\underline{H}$], 135.2 and 135.6 [C$\underline{}$(6)H and benzoyl *p*-C$\underline{H}$], 141.3 [pentafluorophenyl], 143.6 and 143.7 [Fmoc aromatic C$\underline{}$], 149.7 [C$\underline{}$(2)], 155.8 [Fmoc C$\underline{}$O rotamers], 162.3 [C$\underline{}$(4)], 167.4 [benzoyl C$\underline{}$O], 168.5 [peptide C$\underline{}$O], 170.9 [ester C$\underline{}$O]; ν_{max} (KBr)/cm^{-1} 1798s, 1755s, 1713s and 1666s (C=O).

Peptide Synthesis

Syntheses of PNAs containing D- and L-seryl-proline backbone were performed on Novasyn TGR resin [preloaded with Fmoc-L-Lys(Boc)-OH 0.23 mmol/g; 25 mg *ca.* 5 µmol] using the dipeptide pentafluorophenyl esters (8a) or (8b) in the presence of HOBt.H_2O in DMF (4 eq each, 3 h, rt) as described previously.[28] Double coupling was performed followed by end-capping (Ac_2O 18.6 µL, DIEA 27 ?L in DMF 155 ?L) and washing with DMF before removal of the *N*-terminal Fmoc group by treatment of 20 % piperidine in DMF for 15 min. The cycle was repeated until the addition of the final residue was completed, then the PNAs were released from the resin (with the Fmoc group still attached to the *N*-terminus) by treatment with trifluoroacetic acid containing 5% thioanisole (*ca.* 1 mL for a 5 µmol synthesis) at room temperature for 3 h with occasional agitation. The cleavage solution was diluted with diethyl ether (ten times the volume) and kept at -20°C overnight. The suspension was centrifuged and crude PNA was repeatedly washed with ether. Finally the crude PNA was air dried and purified by reverse phase HPLC (for conditions see Fig 1a). The Fmoc group in the purified samples was removed by a brief treatment with 20 % piperidine in DMF (20-50 µL/50 µg). The precipitated Fmoc-OFF peptide was isolated by centrifugation and washed a few times with ether and finally air dried before purification by reverse phase HPLC on a C-18 column eluted with gradient system of 0.1 % aqueous trifluoroacetic acid - acetonitrile.

T_m Studies

The sample for T_m measurement was prepared by mixing calculated amounts of stock oligonucleotide and PNA solutions. The following molar extinction coefficients (ε) were used without compensation for the hypochromic effect due to the formation of ordered secondary structure of single-stranded nucleic acids : A, 15.4 mL.?mol^{-1}.cm^{-1}; T, 8.8 mL.µmol^{-1}.cm^{-1}. The OD_{260} was recorded on a Varian CARY 13 UV spectrophotometer equipped with a temperature controller in steps from 5-95°C (heater temperature) with a temperature increment of 0.25-0.5°C /min. The results were normalized by dividing the absorbance at each temperature by the initial absorbance.

Modelling studies

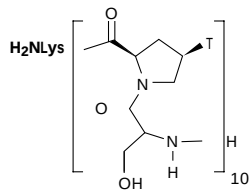
The energy minimized structures of PNA-DNA hybrids were achieved in MacroModel 5.5[30] running on a Silicon Graphics Indigo by generating the DNA duplex sequence in an idealized B-DNA structure, then replacing each atom along the sugar phosphate backbone by its PNA equivalent. For example, the phosphorus atoms were replaced by the serine ?-carbon atoms, etc). The resulting structure was energy minimized using the Batchmin 5.5 software with the AMBER force field[31,32] in an implicit water environment (the dielectric constant of water was used for the calculations) for 1,500 iterations.

Results and Discussion

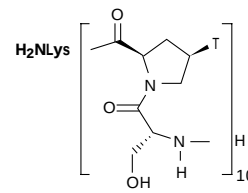
Chemical Synthesis

The target molecules were the thymine-decamers H-[L-Ser-D-Pro(*cis*-4-T)]$_{10}$-LysNH$_2$ (L/D-**4**) and H-[D-Ser-D-Pro(*cis*-4-T)]$_{10}$-LysNH$_2$ (D/D-**4**). The required building blocks for the synthesis of serine-containing PNA were the *C*-terminal activated protected dipeptides (**8a**) and (**8b**) which could be synthesized from the readily available Boc-D-Pro(*cis*-4-*N*^{3}-TBz)-ODpm (**6**) in an analogous manner to the glycylproline analogue (**Scheme 1**).[27] Synthesis of the protected amino acid (**6**) starting from the commercially available *trans*-4-hydroxy-L-proline has been described in the literature.[27,28] Fmoc/^{t}Bu protecting groups were chosen because of the milder conditions for the solid phase peptide synthesis and cleavage from the resin. The Boc group in (**6**) was selectively removed in the presence of the less acid-labile diphenylmethyl ester by treatment with 2.5 equivalents of *p*-toluenesulfonic acid (*p*-TsOH) in acetonitrile. The free amine (as its tosylate) was neutralized with excess diisopropylethylamine (DIEA) and, without isolation, coupled with Fmoc-L-Ser(OtBu)-OH or Fmoc-D-Ser(OtBu)-OH in the presence of dicyclohexylcarbodiimide (DCC) and 1*H*-hydroxybenzotriazole (HOBt) in acetonitrile/*N*,*N*-dimethylformamide. Both enantiomers of protected serine gave similar yields (70-90 %) of the desired dipeptides (**7a** and **7b**), which were isolated as white amorphous solids after column chromatography and were characterized by elemental analysis (C, H, N), ^{1}H, ^{13}C NMR and APCI-MS.

Selective deprotection of the ODpm ester of (**7a**) to the corresponding Fmoc-protected dipeptide free acid was achieved by hydrogenolysis under standard conditions (H_2/Pd-C) which gave complete cleavage of the diphenylmethyl group within 1 hr. Under these conditions, the Fmoc group was not significantly affected. Without purification, the Fmoc-dipeptide free acid was converted to its pentafluorophenyl ester (**8a**) by treatment with pentafluorophenol/DCC and purified by column chromatography on silica gel. A similar yield of (**8b**) was obtained when the same reaction sequence was applied to the ODpm ester containing D-serine (**7b**). Both products gave the expected microanalytical and spectroscopic data.



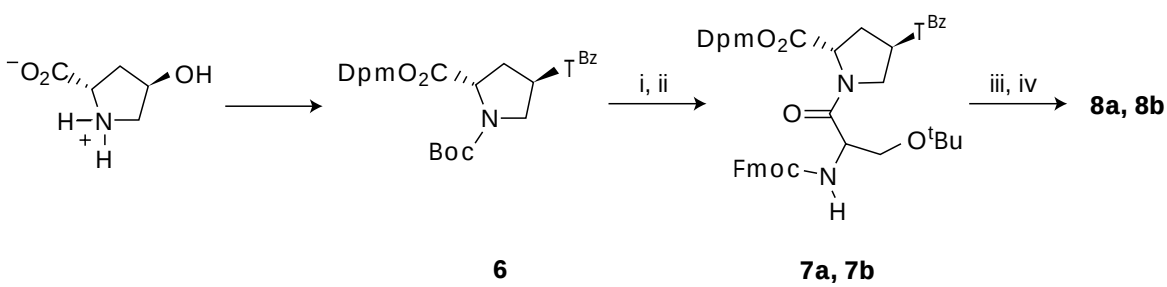
L/D-4



D/D-4

Solid phase synthesis of the decamers, L/D-**4** and D/D-**4** were carried out according to the previously reported protocol[28] on 5 µmol scales. Lysine was included at the C-termini to reduce self aggregration of the uncharged PNA[5,6] and for comparison with previously synthesized PNA which always have lysine attached. The efficiency of each coupling step was monitored by measurement the absorbance of the dibenzofulvene-piperidine adduct released from deprotection of the Fmoc group at 264 or 297 nm and this showed that the coupling proceeded efficiently (95-100%). Capping was performed after each step of the solid phase. The products were cleaved from the resin using 5% thioanisole in TFA while still having the Fmoc group attached at *N*-termini. After purification by reverse phase HPLC, the Fmoc group was removed by treatment with 20% piperidine in DMF and the resulting free peptides were purified again by HPLC. Both PNAs gave single

Scheme 1



6 **7a, 7b**

i 5 eq, p-TsOH, rt, 3h
ii Fmoc-Ser(O^{t}Bu)-OH, DCC, HOBt, DIEA in MeCN:DMF 1:1 rt, 3h
iii 4 N HCl in dioxane:dichloromethane 2:1, rt, O/N
iv PfpOH, DCC in dichloromethane, rt, 2h

Fig 1a Fig 1b

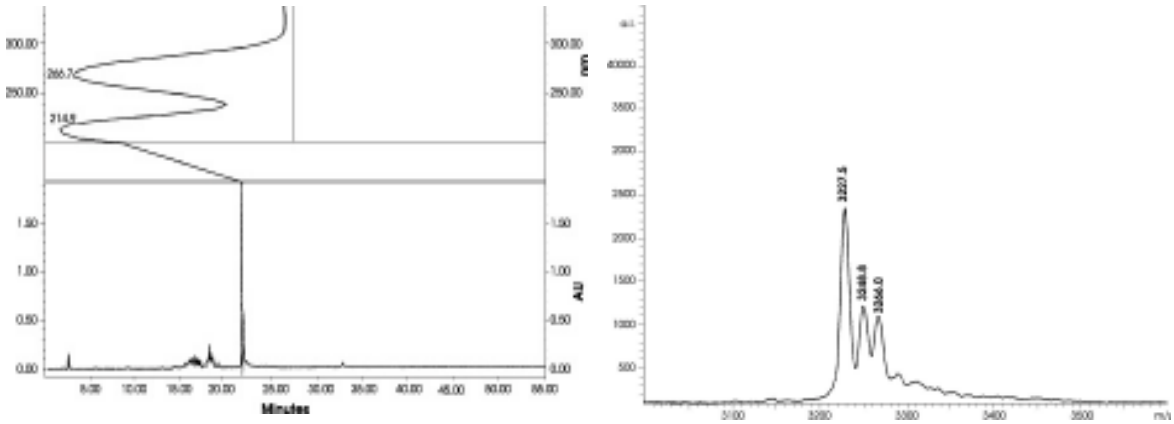


Fig 1. a) Reverse phase HPLC chromatogram of crude Fmoc-ON L/D-4 HPLC conditions: solvents - 0.1% TFA in acetonitrile (A) and 0.1% TFA in water (B) conditions - A:B isocratic 1:99 for 5 min then linear gradient from 1:99 to 90:10 over 35 min, flow rate 1.0 mL/min; Detection wavelength 254 nm. b) MALDI-TOF mass spectra of purified L/D-4

Table 1. Yield and physical data of serine-modified PNA, HPLC conditions: as in legend to **Fig 1.**

PNA	t_R (min)	yield[a] (%)	observed M_r (MALDI-TOF)
L/D-**4**	14.2	28	3227.5 ($M+H^+$) 3248.8 ($M+Na^+$) 3266.0 ($M+K^+$)
D/D-**4**	14.0	16	3227.9 ($M+H^+$) 3249.5 ($M+Na^+$) 3265.7 ($M+K^+$)

[a] calculated yield based on the Fmoc group chromophore released after final deprotection.

peaks by HPLC analysis and gave correct masses by MALDI-TOF (3227.5, $M.H^+$ together with Na^+ and K^+ adducts, Fig 1 and Table1). The purified PNAs L/D-**4** and D/D-**4** were obtained in 28 and 16 % overall yield respectively. Both serine-containing PNAs were freely soluble in water and a concentration of 2 mM could be achieved easily.

Binding Studies

Binding of the two hydrophilic PNAs, L/D-**4** and D/D-**4**, to their complementary oligodeoxyribonucleotide were studied by measuring the absorbance change at 260 nm of 1:1 mixtures of the PNAs and $(dA)_{10}$ at different temperatures. No sharp melting, but a gradual and continuous increase in the absorbance at 260 nm was observed in both cases. Heating of the $(dA)_{10}$ strand in the absence of the PNA gave a similar increase in the absorbance at 260 nm due to the unstacking of the $(dA)_{10}$ strand itself while heating of single stranded L/D-**4** alone gave no significant change in the absorbance.

Polyacrylamide gel electrophoresis confirmed the lack of binding of D/L-**4** and D/D-**4** to fluorescently labelled $(dA)_{10}$ since no new species was formed even at high PNA:DNA ratio whereas a positive control experiment using Nielsen's aminoethylglycine PNA (1) H-(aeg-T)$_8$-Lys-NH_2 showed a distinct slow moving band due to the $(PNA)_2$•DNA hybrid. Thus it appears that no hybridization between the serine-modified PNAs and $(dA)_{10}$ had taken place.

The lack of binding of both L/D-4 and D/D-4 with $(dA)_{10}$ is quite unexpected. It is not clear why incorporation of a relatively small, hydrophilic side-chain of serine into PNA would make them totally lose the ability to hybridize with complementary oligodeoxynucleotides. Steric requirement of the serine side-chain is clearly not negligible as it was previously reported that substitution of the glycine in PNA (1) with several other amino acids resulted in PNA with poorer binding properties (ΔT_m in the range of -5 to -7°C per modification).[15,16] Recently, it has been found that some PNA show a tendency to form ordered secondary structures *via* base-pairing and/or backbone interactions. For example, Diederichsen and Schmitt[33] showed that oligoadenine PNA β-homoalanyl PNA [H-(β-HalA)$_n$-LysNH_2] can form quite stable self-pairing aggregrates *via* non-classical A•A pairing even in the presence of lysinamide at the C-terminus. It is conceivable that under favourable circumstance, wobble T•T pairing is also possible and this might result in aggregration of oligo-T PNA. In addition, the serine hydroxyl side chain which has the potential to act as a hydrogen bond donor and

acceptor in serine-modified PNA may result in stable secondary structures *via* hydrogen bond formation, either intramolecularly or intermolecularly.

Comparison of energy-minimized molecular models of hybrids formed between a mixed sequence DNA with complementary glycyl-D-proline PNA and L-seryl-D-proline PNA have been made. The energy minimized structure of the hybrid with serine-modified PNA and DNA is seriously distorted from the optimal B-DNA conformation compared to the original glycine-containing PNA. However, care should be taken in attempting to draw conclusions from such models since it is known from an NMR and X-ray crystallographic study[12-14] that the real structures of PNA•DNA hybrids are quite different from the originally proposed B-DNA-like models.[10,11]

In conclusion, the study has demonstrated that PNA can be manipulated relatively easily by changing the spacer amino acid and this can affect the properties of the molecules in many ways, sometimes quite unexpectedly. Substituting a glycine spacer by D-serine and L-serine improved the solubility of the resulting PNAs. However, both serine-containing T_{10} PNAs showed no detectable hybridization to $(dA)_{10}$ under the conditions which glycine-containing PNA binds quite strongly.

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