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Synthesis of chiral peptide nucleic acids using Fmoc chemistry

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Abstract—A Fmoc-based synthesis of chiral PNAs is described. Chiral monomer backbones were efficiently prepared by reductive amination of *N*-Fmoc-protected L,D-alaninals with glycine esters and the subsequent acylation of free amines with thymine-1-ylacetic acid. The dimer derivative of L-amino acid was prepared in solution. Finally, a chiral decamer was obtained by a solid phase strategy using a succinyl-linked support.

1. Introduction

Peptide nucleic acids (PNAs) are DNA mimics in which nucleic acid bases are incorporated to a pseudopeptide backbone.[1–3] PNAs recognize their complementary oligonucleotides with a remarkably high specificity and affinity, and are resistant to nucleases and proteases.[4–7] These properties of PNAs have special attention in the development of gene therapeutics and bio-molecular tools. The original PNA backbone consists of *N*-(2-aminoethyl)glycine units, and nucleobases are attached to the glycine nitrogen through methylene carbonyl linkers. Introduction of chirality into the backbone has attracted much attention.[8–10] Such a modification might serve as a model for the introduction of active groups at various positions along the backbone and leads to the improvement of such parameters as binding affinity, specificity and cell permeability. For instance, Lenzi et al. have prepared a PNA mimic involving aminobutyric acid and glycine.[11]They demonstrated that this chiral peptidic DNA exhibited good self-recognition similar to that of DNA–DNA duplexes.

To synthesize chiral PNAs, different methods have been developed, amongst which a Boc protecting group strategy is widely used to realize the oligomerization.[12–14] The repeated use of TFA and the harsh HF or TFMSA treatment mean this strategy will be incompatible with many types of modified PNAs, especially with the PNA–DNA chimera due to the sensitivity of DNA to strong acids. In search of an alternative strategy which may provide a feasible route to the synthesis of PNAs with special groups and oligonucleotides, we developed a simplified and efficient procedure to synthesize *N*-Fmoc protected chiral PNAs (Scheme 1). Herein, the synthesis of the thymine containing chiral PNA monomers from *N*-Fmoc protected L or D-alanine and the subsequent incorporation into a chiral dimer using solution phase techniques and a base-cleavage solid phase strategy for the preparation of chiral PNA are described in detail.

2. Results and discussion

2.1. Monomer synthesis

The chiral monomer backbones can be prepared by reductive amination of the glycine ester with *N*-Fmoc amino aldehydes. This procedure gave *N*-Fmoc protected chiral backbones in good purity and yield. First, *N*-Fmoc L,D-alanines were transformed into their corresponding *N*,*O*-dimethylhydroxyl-amide derivatives[12] (**1a** and **1b**) via mixed anhydrides using isobutylchloroformate (IBCF). Reduction of the amides was performed with five equivalents of hydride (1.25 equiv. of $LiAlH_4$) to produce the corresponding aldehydes **2a**, **2b** within 10–20 min at 0°C. After crystallization, the aldehydes were reacted with glycine ester and the resulting imines were efficiently transformed to amines using two different reductants. The intermediate imines from the ethyl ester of glycine were treated with 10% Pd/C, while the imines obtained from the benzyl ester of glycine were treated with $NaBH_3CN$. *N*-Fmoc protected L,D chiral monomer backbones **3a–d** were obtained in 70–85% yield, respectively, after purification by flash chromatography (Scheme 2). All of these amines were shelf-stable solids which could be stored for more than two weeks. A more attractive method involved transforming the crude materials into the corresponding stable hydrochloride salts **3c**, **3d** by treating with an equivalent of dry ethereal HCl (1.5 M) at −20°C, which can be stored for several months. The optical purities of **3a** and **3b** reached 99.3 and 96% by chiral HPLC measurement, which were similar to the *N*-Fmoc alanine.

Keywords: *N*-Fmoc; glycine; oligomerization.

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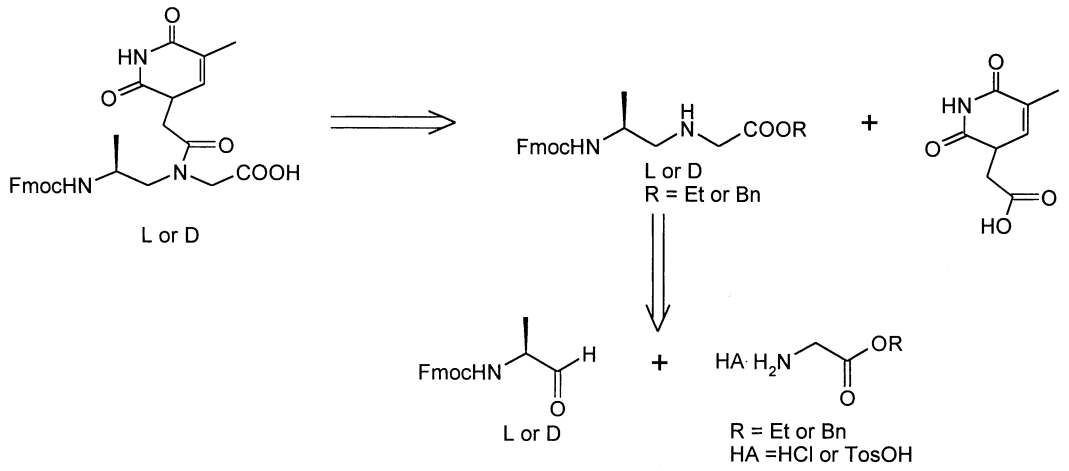
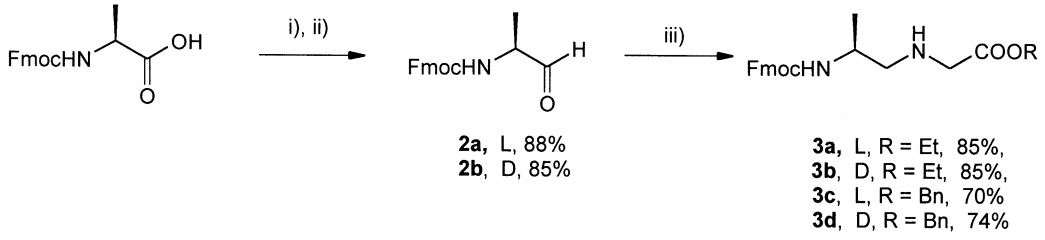


Scheme 1.

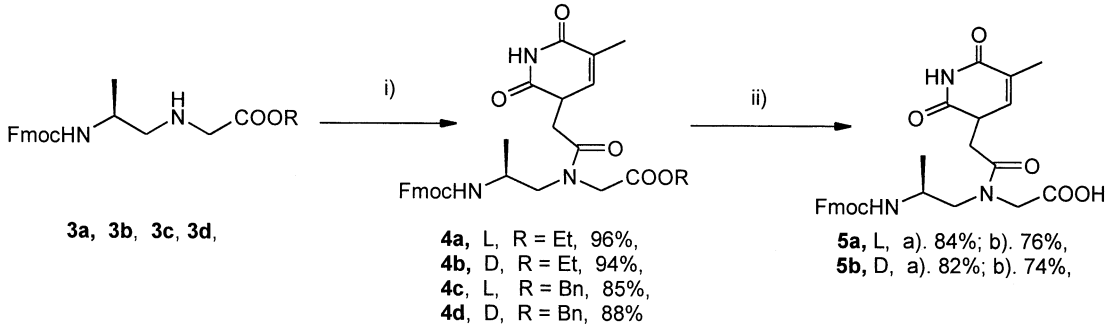


Scheme 2. *Reagents and conditions*: (i) ^{t}BuO–CO–Cl, NMM, THF; HCl·HNMe(OMe), NEt_3; −20°C. (ii) $LiAlH_4$, THF, 0°C. (iii) (a). HCl·NH_2CH_2COOEt, KOAc, 10% Pd/C, MeOH; room temperature. (b). Tos·NH_2CH_2COOBn, HOAc, $NaBH_3CN$, MeOH, 0°C.

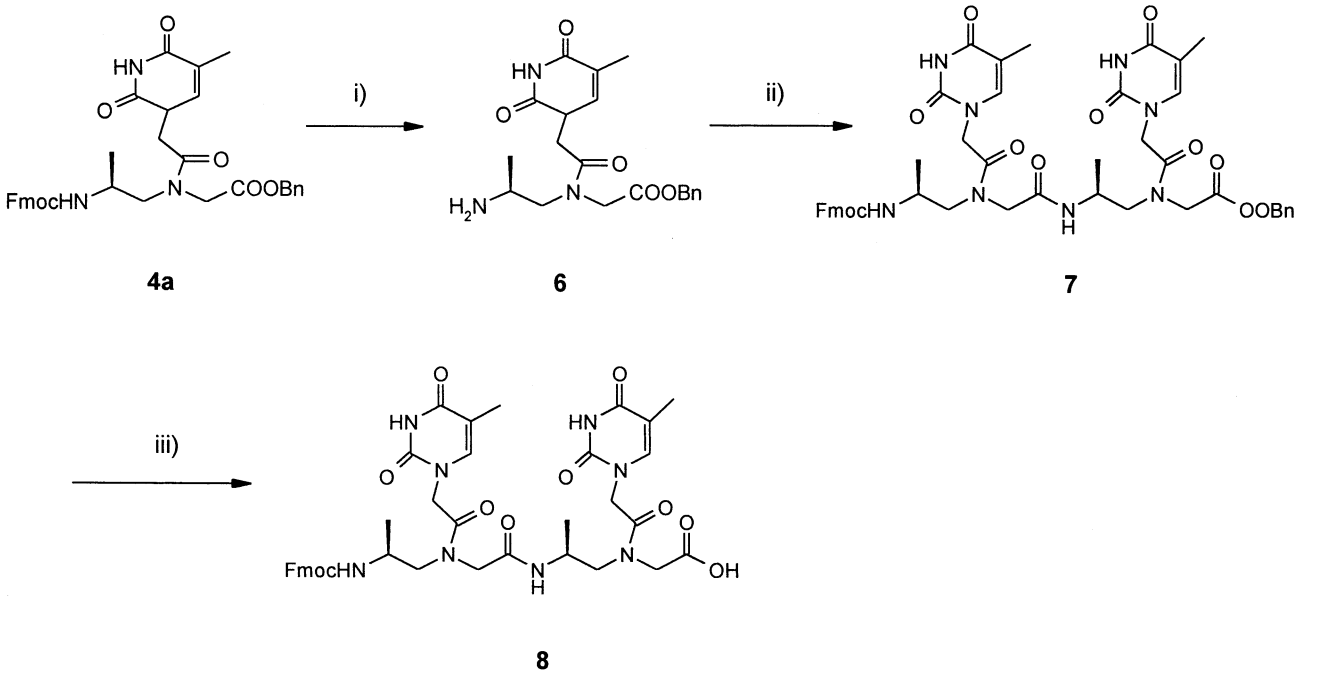
The acylation of free amines with thymin-1-yl acetic acid can be carried out by conventional methods using BBC-Cl[15] as the coupling reagent. **4a**–**4d** were obtained in good to excellent yields (85–95%), though the other uronium or phosphonium reagents were ineffective for this coupling. As shown in Scheme 3, removal of the benzyl ester from **4c** and **4d** was performed by hydrogenation with 10% Pd/C and the ethyl group of **4a** and **4b** was cleaved with NaOH (5 M, 10 equiv.), since ethyl and methyl esters are more sensitive to aqueous NaOH than the Fmoc group at low temperature within a short period of time (5–10 min).[16] The Fmoc protected chiral PNA monomers were obtained after purification by flash chromatography.

2.2. Solution phase oligomerization

Dimerization of *N*-Fmoc protected L-dipeptides was then investigated. The Fmoc group of **4c** was removed easily using a 50% solution of *N*,*N*-diethylamine in DCM to give monomer **6**, which was coupled with **5c** using FEP as the coupling reagent. As expected, FEP[17] gave higher coupling yields than uronium salt (TBTU) and phosphonium salt (BOP) and afforded dimer **7** in 70% yield. Only one product is obtained indicating that no racemization occurred during the coupling. Finally, hydrogenation of **7** with 10% Pd/C in ethanol gave **8** in 85% yield (Scheme 4).



Scheme 3. *Reagents and conditions*: (i) Thymin-1-yl acetic acid, BBC-Cl, DIPEA, DMF/DCM; rt. (ii) (a). R=Bn: 10% Pd/C, EtOH; room temperature. (b). R=Et:NaOH (2.5 M), H_2O–THF, 0°C, within 5–10 min.



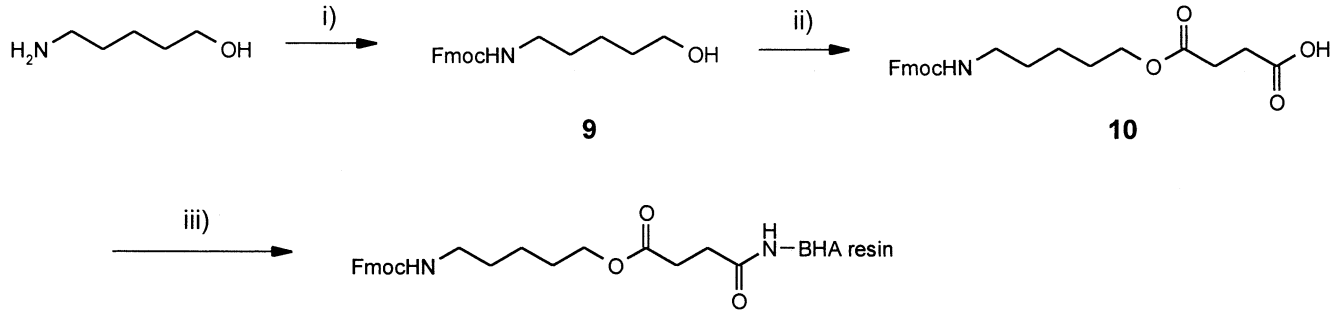
Scheme 4. *Reagents and conditions*: (i) 50% $HNEt_2$ in DCM, 30 min. (ii) **5a**, FEP, DIPEA, −10°C. (iii) 10% Pd/C, EtOH; room temperature.

2.3. Solid phase oligomerization

Solid phase peptide synthesis using Boc methodology requires exposure of the peptide resin to strong acids for each cycle. These conditions are incompatible with an orthorgonal strategy and will be unsuitable for the synthesis of phosphono-PNAs because of their acid sensitivity. In 1982, Apsimon et al. developed a new solid support to synthesis β-aminoalcohol, which is cleavable under base treatment.[18,19] We introduced this support with 5-amino-pentasuccinate linker into our Fmoc based peptide synthesis. Thus, 5-aminopentan-1-ol **9** was selectively protected using Fmoc-OSu to give **10**. The resultant alcohol was succinylated using succinic anhydride and DMAP in DCM to give **11**, which was then coupled to BHA resin using AOP/DIPEA in DCM (Scheme 5).

The chiral decathymine PNA with D configuration was successfully prepared by the successive coupling of the corresponding monomers. The PNA was synthesized on a 40 μmmol scale. First, deprotection of the Fmoc group was realized with 20% piperidine in DMF. Then the coupling of the resultant amine with 2.5 equiv. of the Fmoc protected amino acid **5b** was conducted in the presence of AOP, HOAt and DIPEA in anhydrous DMF/DCM. For this, the reagents are pre-mixed in the delivery syringe, and after a short preactivation period delivered to the reaction vessel. After 2 h of coupling, unreacted amino functions were capped using a mixture of 5% acetic anhydride in DMF, which is used as a capping reagent in standard Fmoc strategy SPPS.

The efficiency of reaction is judged by measuring the absorbance of the dibenzofulvene–piperidine adduct (ξ_{264} 18,000 $dm^3\ mol^{-1}\ cm^{-1}$), liberated during deprotection. Finally, the PNA is cleaved from the solid support and deprotected with 0.5N $NaOH/H_2O$/THF solution, followed by 50% aqueous acetic acid for the mixture. After de-salting by gel filtration, the chiral PNA was purified by RP-HPLC using a C18 column and a linear gradient acetonitrile (from 20 to 23%) in water with 0.05% TFA. The identity and purity of the oligomer was determined by ESI-MS (Fig. 1). These oligomers showed an ability to form adducts with alkali metal ions especially potassium. The principal mass peak at 2946.2 Da corresponds to the potassium salt of the desired PNA oligomer ($C_{125}H_{173}N_{41}O_{41}$: M=2906) and



Scheme 5. *Reagents and conditions*: (i) Fmoc-OSu, DIPEA, DCM. (ii) Succinic anhydride, DMPA, DCM/Py (3:1), 0°C, room temperature. (iii) BHA resin, AOP, DIPEA, DMF/DCM, −10°C.

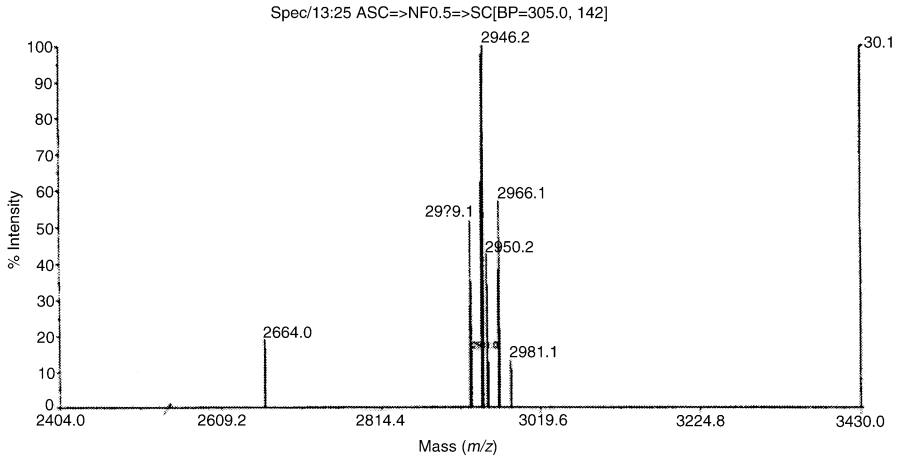


Figure 1. ESI mass spectrum of the pure oligomer.

the other one at 2929.1 Da to the sodium salt of the oligomer (i.e. $[M+Na]^+$).

3. Conclusions

A Fmoc based strategy was successfully applied to the synthesis of chiral PNAs. In this synthesis, chiral monomers were prepared using a reductive amination method. Subsequent connection of the backbones with nucleobases was accomplished using BBC-Cl as the coupling reagent, and the dimerization was carried out in solution using FEP as coupling reagent. A modified amino linker resin was then used as the support to accomplish the Fmoc-based solid phase synthesis of a chiral PNA under mild base conditions.

4. Experimental

Melting points were taken on digital melting point apparatus and uncorrected. Infrared spectra were recorded on a Shimadzu IR-440 spectrometer. Mass spectra were recorded on HP 5989A and VG QUATTRO mass spectrometers. ^{1}H NMR spectra were recorded on Bruker AM 300 (300 MHz) and Bruker DRX-400 (400 MHz) using TMS as internal standard. Combustion analysis for elemental composition was carried out on an Italy MOD 1106 analyzer. Optical rotations were determined using a Perkin–Elmer 241 MC polarimeter. Flash column chromatography was performed with 300–400 mesh silica gel, and analytical thin layer chromatography was performed on pre-coated silica gel plates (60F-254) with the systems (v/v) indicated. Solvents and reagents were purified by standard methods as necessary. Amino acids were L-configuration if not otherwise stated. HOAt, AOP, BOP, TBTU, DIPEA, thymine, *N*,*O*-dimethylhydroxylamine hydrochloride, $LiAlH_4$, BHA resin were purchased from Aldrich Chemical Co, WI, and used without purification.

4.1. Synthesis of compounds

4.1.1. *N*-[(9*H*-Fluoren-9-ylmethoxy)carbonyl]-L or D-alanyl-*N*-methoxy-*N*-methylamide (1a or 1b). *N*-Fmoc-L or D-alanine (6.224 g, 20 mmol) and *N*-methylmorpholine (22 mL, 20 mmol) were dissolved in 120 mL of dry THF and cooled to −20°C. Then, isobutylchloroformate (2.6 mL, 20 mmol) was added in one portion under stirring and left for several minutes. Triethylamine (3.06 mL, 22 mmol) was added followed by a solution of *N*,*O*-dimethyl-hydroxylamine hydrochloride (1.94 g, 20 mmol) in 50 mL of dry DMF, which had been chilled to the same temperature. After stirring for 30 min at −20°C, the reaction mixture was allowed to warm to room temperature. The precipitates were removed by filtration, the filtrate was diluted with 200 mL of water and 20 mL of 1N HCl. After evaporation in vacuo, the solution was adjusted to pH 8 with 2N K_2CO_3 and then cooled in freezer overnight. The precipitates were collected by filtration, briefly washed with deionized water, and dried in vaccum at 40°C for 3 h.

Compound **1a** was obtained as crystalline solid, yield 92%, mp: 120–121°C (dec.), $[\alpha]_D^{20}=+2.1$ (*c* 0.68 $CHCl_3$).[20] ^{1}H NMR (90 MHz, $CDCl_3$) δ 7.85–7.2 (8H, m, Fmoc-aromatic-C*H*), 5.6 (1H, m, N*H*), 4.75–4.20 (4H, m, Fmoc-aliphatic-C*H* and $-CH_2$ and $-CH$Me), 3.75 (3H, s, OCH_3), 3.2 (3H, s, NCH_3), 1.35 (3H, d, *J*=5.4 Hz, $-CHCH_3$).

Compound **1b** was obtained as crystalline solid, yield 91%, mp: 124–125°C (dec.), $[\alpha]_D^{20}=-2.33$ (*c* 1 $CHCl_3$).[20] ^{1}H NMR (90 MHz, $CDCl_3$) δ 8.0–7.3 (8H, m, Fmoc-aromatic-C*H*), 5.70 (1H, m, N*H*), 5.0–4.3 (4H, m, Fmoc-aliphatic-C*H* and-CH_2 and $-CH$Me), 3.80 (3H, s, $-OCH_3$), 3.20 (3H, s, NCH_3), 1.30 (3H, d, *J*=5.4 Hz, $-CH_3$).

4.1.2. *N*-[(9*H*-Fluoren-9-ylmethoxy)carbonyl]-L or D-alaninal (2a or 2b). A solution of **1a** or **1b** (5.31 g, 15 mmol) in 80 mL of dry THF was cooled to 0°C, and then $LiAlH_4$ (0.69 g, 18.8 mmol) was added. The reaction was followed by TLC mixture until completion. A solution of $KHSO_4$ (5.44 g, 40 mmol) was added and the reaction mixture was extracted with ethyl acetate (3×50 mL). The combined organic extracts were washed with 1N HCl (2×50 mL), $NaHCO_3$ (2×50 mL) and brine (2×50 mL), dried and evaporated to give a white solid. Pure product was obtained by recrystallization from petroleum–ethyl acetate.

Compound **2a**, white solid, yield 95%, mp:137–138°C (lit.[21] mp:145°C), mp:147–148°C (lit.[21] mp:145°C), $[\alpha]_D^{20}=43.4$ (*c* 1 $CHCl_3$) [lit.[21] $[\alpha]_D=10$ (*c* 1 $CHCl_3$)]. ^{1}H NMR

(90 MHz, $CDCl_3$) δ 9.5 (1H, s, C*H*O), 7.85–7.20 (8H, m, Fmoc-aromatic-C*H*), 5.40 (1H, m, N*H*), 4.40–4.10 (4H, m, Fmoc-aliphatic-C*H* and $-CH_2$ and $-CH$Me), 1.30 (3H, d, J=5.4 Hz, $-CH_3$).

Compound **2b**, white solid, yield 93%, mp:136–137°C (lit.[21] mp:145°C), $[\alpha]_D^{20}=-44.1$ (c 1.08 $CHCl_3$) [lit.[21] $[\alpha]_D=-11$ (c 1 $CHCl_3$)]. ^{1}H NMR (90 MHz, $CDCl_3$) δ 9.45 (1H, s, C*H*O), 7.85–7.15 (8H, m, Fmoc-aromatic-C*H*), 5.40 (1H, m, N*H*), 4.40–4.15 (4H, m, Fmoc-aliphatic-C*H* and $-CH_2$ and $-CH$Me), 1.25 (3H, d, J=5.4 Hz, $-CH_3$).

4.2. Reductive amination of aldehydes and glycine ester

4.2.1. Ethyl *N*-(2-Fmoc-amino-L or D-methylethyl)glycinate (3a or 3b). A solution of *N*-Fmoc-alaninal **2a** or **2b** (4.25 g, 14.4 mmol) in methanol (100 mL) was added to a solution of glycine ethyl ester hydrochloride (2.008 g, 14.4 mmol) and anhydrous CH_3COOK (2.83 g, 28.9 mmol) in methanol (150 mL). The mixture was cooled to 0°C under nitrogen for 10 min and 10% Pd/C (46 mg) was added with vigorous stirring. The reaction mixture was hydrogenated at atmospheric pressure and room temperature until hydrogen uptake had ceased. The catalyst was filtered off and solvent was evaporated under reduced pressure. The residue was suspended in water (25 mL) and the pH was adjusted to 8 by addition of 1N NaOH. The aqueous phase was extracted with DCM (5×25 mL), the organic layer was washed with $NaHCO_3$ (2×25 mL), brine (2×25 mL), and dried (Na_2SO_4). After evaporation of the solvent, the crude product was purified by flash chromatography.

Compound **3a**, white solid, yield 85%, mp: 85.5–87°C (dec.), $[\alpha]_D^{16}=5.55$ (c 0.88 $CHCl_3$). [Found: C, 68.92; H, 6.69; N, 7.24%. $C_{22}H_{26}N_2O_4$ requires C, 69.09; H, 6.85; N, 7.32%]; ν_{max}(KBr): 3323 (NH), 1737, 1686 (C=O), 759, 739 (Fmoc) cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.77–7.26 (8H, m, Fmoc-aromatic-C*H*), 5.21 (1H, s, FmocN*H*), 4.39 (2H, d, J=6.6 Hz, Fmoc-aliphatic-CH_2), 4.25–4.02 (3H, m, Fmoc-aliphatic-C*H*, CH_2Me), 3.81 (1H, m, $-CH$Me), 3.41 (2H, d, J=7.2 Hz, HNCH_2CO_2Et), 2.62 (2H, d, J=1.1 Hz, CHCH_2NH), 1.97 (1H, s, CH_2NHCH_2), 1.26 (3H, t, J=6.0 Hz, CH_2CH_3), 1.18 (3H, d, J=5.3 Hz, CH CH_3); ESIMS m/z: 383.3 $[M+H]^+$, 100%, 765 $[2M+H]^+$, 8%, 178 $[Fmoc]^+$, 44%; ee=99.3%.

Compound **3b**, white solid, yield 85%, mp: 86–87°C (dec.), $[\alpha]_D^{16}=-5.96$ (c=0.869 in $CHCl_3$). [Found: C, 68.91; H, 6.65; N, 7.21%. $C_{22}H_{26}N_2O_4$ requires C, 69.09; H, 6.85; N, 7.32%]; ν_{max}(KBr): 3321 (NH), 1739, 1685 (C=O), 759, 739 (Fmoc) cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.76–7.29 (8H, m, Fmoc-aromatic-C*H*), 5.21 (1H, s, FmocN*H*), 4.39 (2H, d, J=6.5 Hz, Fmoc-aliphatic-CH_2), 4.25–4.16 (3H, m, Fmoc-aliphatic-C*H*, CH_2Me), 3.83 (1H, m, C*H*Me), 3.43 (2H, d, J=7.6 Hz, HNCH_2CO_2Et), 2.70 (2H, d, J=3.6 Hz, CHCH_2NH), 2.25 (1H, s, CH_2NHCH_2), 1.28 (3H, t, J=7.1 Hz, CH_2CH_3), 1.19 (3H, d, J=5.7 Hz, $CHCH_3$); EIMS m/z: 383 $[M+H]^+$, 2%, 178 $[Fmoc]^+$, 85%, 165 $[M\text{-}Fmoc\text{-}CO_2]^+$, 100%; ee=96%.

4.2.2. Benzyl *N*-(2-Fmoc-amino-L or D-methylethyl)glycinate (3c or 3d). A pre-cooled 10% solution of benzyl glycinate toluene-*p*-sulfonate (3.71 g, 11 mmol) in methanol (40 mL) was treated with **2a** or **2b**. The suspended solution was stirred at 0°C for 30 min, to which acetic acid (0.75 mL) and $NaCNBH_3$ (0.37 g, 5.9 mmol) were added sequentially and left for 2 h, the solvent was removed under reduced pressure to give a colorless oil. The oil was dissolved in 200 mL of ethyl acetate and 80 mL of 5% $NaHCO_3$. The aqueous layer was separated and extracted with ethyl acetate (3×100 mL), the combined organic layer was washed successively with 5% $NaHCO_3$ (3×80 mL) and brine (3×80 mL) and dried with Na_2SO_4. The solvent was evaporated and the residue was treated with dry ethereal HCl (1.5 M) and precipitated by dry hexane (80 mL). The resultant solid hydrochloride salt was collected by filtration. The pure product was given as white solid after recrystallization from methanol–ether–hexane.

Compound **3c**, white solid, yield 70%, mp: 180–181°C (dec.). [Found: C, 66.71; H, 6.02; N, 5.75. $C_{27}H_{28}N_2O_4{\cdot}HCl{\cdot}1/4H_2O$ requires C, 66.80; H, 6.12; N, 5.77%]; ν_{max}(KBr): 3316 (NH), 1746, 1690 (C=O), 759 and 739 (Fmoc and Ph) cm^{-1}; ^{1}H NMR (300 Hz, $CDCl_3$) δ 9.80 (1H, m, N*H*·HCl), 7.73–7.27 (13H, m, phenyl- and Fmoc-aromatic-C*H*), 6.54 (1H, s, FmocN*H*), 5.11 (2H, s, CH_2Ph), 4.63–4.12 (4H, m, Fmoc-aliphatic-C*H* and $-CH_2$ and $-CH$Me), 3.96 (2H, s, HNCH_2CO), 3.42–3.21 (2H, m, CHCH_2NH), 1.26 (3H, m, $CHCH_3$); ESIMS m/z: 445 $[M+H]^+$, 100%, 178 $[Fmoc]^+$, 42%, 91 $[Bn]^+$, 35%.

4.2.3. Acylation of the pseudodipeptide with thymin-1-ylacetic acid. Amine **3a**–**3c** or **3d** (10 mmol), thymin-1-ylacetic acid (2.02 g, 11 mmol) and BBC-Cl (3.66 g, 11 mmol) were suspended in 10 mL of dry DMF and 5 mL of dry DCM at 0°C and stirred for a few minutes, then DIPEA (3.84 mL, 22 mmol) was added at the same temperature. The mixture was allowed to warm to room temperature with stirring overnight, the solvent was evaporated and the residue was partitioned between water (50 mL) and ethyl acetate (50 mL), the aqueous phase was extracted with ethyl acetate (4×50 mL). The combined organic extract was washed with 1N HCl (2×50 mL), $NaHCO_3$ (2×50 mL) and brine (2×50 mL), and then dried with Na_2SO_4. The solvent was evaporated to dryness and the crude products were purified by flash chromatography (hexane–ethyl acetate, 1:2) to give the desired products.

Compound **4a**, white solid, yield 95%, mp: 179–180°C. [Found: C, 63.20; H, 6.17; N, 10.16%. $C_{29}H_{32}N_4O_7$ requires C, 63.49; H, 5.88; N, 10.41%]; ν_{max}(KBr): 1682 (C=O), 760 and 739 (Fmoc) cm^{-1}; ^{1}HNMR (400 MHz, d_6-DMSO) two isomers δ 11.28 and 11.27 (1H, 2×s, thymine-N*H*(3)), 7.90–7.31 (8H, m, Fmoc aromatic-C*H*), 7.25 (1H, s, thymine-C*H*(6)), 4.76–3.0 (8H, m, C*H*Me, Fmoc aliphatic-C*H* and $-CH_2$, NCH_2CO, CH_2CO, CHCH_2N and CH_2Me), 1.73 and 1.70 (3H, 2×s, thymine-CH_3), 1.20 (3H, 3×t, J=7.0 Hz, CH_2Me), 1.05 (3H, 2×d, J=6.3 Hz, CH_3); ESI-MS m/z: 549.4 $[M+H]^+$, 100%, 571.4 $[M+Na-H]^+$, 90%, 587.4 $[M+K-H]^+$, 20%.

Compound **4b**, white solid, yield 94%, mp 179–180°C. [Found: C, 63.05; H, 6.19; N, 10.16%. $C_{29}H_{32}N_4O_7{\cdot}1/4H_2O$ requires C, 62.98; H, 5.92; N, 10.13%]; ν_{max}(KBr): 1743 and 1682 (C=O), 760 and 739 (Fmoc) cm^{-1}; ^{1}H NMR (400 MHz, d_6-DMSO) two isomers δ 11.28 an 11.27 (1H,

2×s, thymine-N*H*(3)), 7.90–7.18 (8H, m, Fmoc aromatic-C*H*), 7.25 (1H, s, thymine-C*H*(6)), 4.70 (1H, q, *J*=7.7 Hz, C*H*Me), 4.47–4.0 (7H, m, Fmoc aliphatic-C*H* and –CH_2, CH_2Me and CH_2CO), 4.09–3.00 (4H, m, CHCH_2N and NCH_2CO), 1.73 and 1.71 (3H, 2×s, thymine-CH_3), 1.20 (3H, 2×t, *J*=7.0 Hz, CH_2Me), 1.05 (3H, 2×d, *J*=6.3 Hz, CH_3); ESI-MS *m/z*: 549.4 $[M+H]^+$, 21%, 571.4 $[M+Na-H]^+$, 100%, 587.4 $[M+K-H]^+$, 28%.

Compound **4c**, white solid, yield 85%, mp: 204–205°C (dec.), $[\alpha]_D^{20}=+9.0$ (*c* 0.88 in DMF). [Found: C, 63.18; H, 5.31; N, 8.93%. $C_{34}H_{34}N_4O_7\cdot 2H_2O$ requires C, 63.15; H, 5.92; N, 8.66%]; ν_{max}(KBr): 1713, 1712 (C=O); 756,737 and 697 (Fmoc and -Ph) cm^{-1}; ^1H NMR (300 MHz, d_6-DMSO) two isomers δ 11.30 and 11.28 (1H, 2×s, thymine-N*H*(3)), 7.89–7.29 (13H, m, phenyl and Fmoc-aromatic-C*H*), 7.23 and 7.17 (1H, 2×s, thymine-C*H*(6)), 5.20 and 5.12 (2H, 2×s, CH_2Ph), 4.71 (1H, m, C*H*Me), 4.49–3.00 (9H, m, Fmoc-aliphatic-C*H* and –CH_2 and –CH_2CO and CH_2NCH_2), 1.73 and 1.71 (3H, 2×s, thymine-CH_3), 1.05 (3H, 2×d., *J*=6.3 Hz, CHCH_3); ESIMS *m/z*: 633 $[M+Na]^+$, 100%, 1233 $[2M+Na]^+$, 55%, 1855 $[3M+Na]^+$, 34%.

Compound **4d**, white solid, yield 88%, mp: 204–205°C (dec.). [Found: C, 66.75; H, 5.78; N, 9.08%. $C_{34}H_{34}N_4O_7$ requires C, 66.87; H, 5.61; N, 9.17%]; ν_{max}(KBr): 1732, 1712 and 1687 (C=O); 756,737 and 698 (Fmoc and -Ph) cm^{-1}; ^1H NMR (300 MHz, d_6-DMSO) two isomers δ 11.13 (1H, brm, thymine-N*H*(3)), 7.90–7.18 (13H, m, phenyl and Fmoc-aromatic-C*H*), 7.09 and 7.08 (1H, 2×s, thymine-C*H*(6)), 5.20 and 5.12 (2H, 2×s, CH_2Ph), 4.72 (1H, q, *J*=7.1 Hz, C*H*Me), 4.50–4.19 (5H, m, Fmoc-aliphatic-C*H* and –CH_2 and –CH_2CO), 4.12–3.05 (4H, m, CH_2NCH_2), 1.73 and 1.71 (3H, 2×s, thymine-CH_3), 1.05 (3H, 2×d, *J*=6.3 Hz, CHCH_3); ESIMS *m/z*: 611 $[M+H]^+$, 100%, 1221 $[2M+H]^+$, 75%.

4.2.4. *N*-(2-Fmoc-Amino-2-L or D-methylethyl)-*N*-(thymin-1-ylacetyl)glycine (5a or 5b). (a) 5 M Sodium hydroxide (2 mL) was added to a stirred solution of **4a** or **4b** (0.549 g, 1 mmol) in THF (2 mL) at 0°C. The completion of reaction was monitored by TLC (within 5–10 min). Water (10 mL) was added and the pH was adjusted to 7–8 by the addition of 1N HCl. The aqueous phase was extracted with $CHCl_3$ (3×10 mL), the pH was adjusted to 2 and extracted with ethyl acetate (8×20 mL). The organic layer was dried over Na_2SO_4 and evaporated to give the crude product. After crystallization or purification by flash chromatography (ethyl acetate–methanol–water 5:1:0.2), the desired product was obtained.(b) **4c** or **4d** (0.305 g, 0.5 mmol) was suspended in 200 mL of ethanol. After refluxing for 20 min, the solution was cooled to room temperature and 10% Pd/C (50 mg) was added under nitrogen and hydrogenated until the substrate disappeared (by TLC). The reaction mixture was filtered and the solvent was evaporated. The crude product was purified by flash chromatography (ethyl acetate–methanol–water 5:1:0.2).

Compound **5a**, white solid, yield 76%, mp: 197–198°C, HPLC 99.7%. [Found: C, 56.77; H, 5.52; N, 10.23%. $C_{27}H_{28}N_4O_7\cdot 3H_2O$ requires C, 56.44; H, 5.96; N, 9.75%]; ν_{max}(KBr): 3334 (OH), 1682 (C=O); 761,739 (Fmoc) cm^{-1}; ^1H NMR (300 MHz, d_6-DMSO) two isomers δ 11.28 and 11.24 (1H, 2×s, thymine-N*H*(3)), 7.90–7.25 (9H, m, Fmoc-aromatic-C*H* and T-C*H*(6)), 4.71 (1H, m, –C*H*Me), 4.46–3.58 (9H, m, Fmoc-aliphatic-C*H* and –CH_2 and –CH_2NCH_2 and CH_2CO and H_2O), 1.72 and 1.71 (3H, 2×s, thymine-CH_3), 1.05 (3H, 2×d, *J*=6.4 Hz, CHCH_3); ESIMS *m/z*: 521 $[M+H]^+$, 10%, 543 $[M+Na]^+$, 100%, 1064 $[2M+Na]^+$, 75%, 1584 $[3M+Na]^+$, 15%.

Compound **5b**, white solid, yield 80%, mp: 202–203°C; $[\alpha]_D^{16}=-10.02$ (*c* 1.08 DMF), HPLC 99.3%. [Found: C, 60.77; H, 5.63; N, 10.00%. $C_{27}H_{28}N_4O_7\cdot H_2O$ requires C, 60.22; H, 5.61; N, 10.40%]; ν_{max}(KBr): 3316 (OH), 1688 (C=O), 760, 738 (Fmoc) cm^{-1}; ^1H NMR (300 MHz, d_6-DMSO) two isomers δ 11.32 and 11.28 (1H, 2×s, thymine-N*H*(3)), 7.91–7.23 (9H, m, Fmoc-aromatic-C*H* and T-C*H*(6)), 4.69 (1H, m, –C*H*Me), 4.53–3.04 (9H, m, Fmoc-aliphatic-C*H* and –CH_2 and CH_2CO and CH_2NCH_2 and H_2O), 1.71 and 1.72 (3H, 2×s, thymine-CH_3), 1.10 (3H, m, CHCH_3); ESIMS *m/z*: 521 $[M+H]^+$, 65%, 543 $[M+Na]^+$, 25%, 1041 $[2M+H]^+$, 100%, 1063 $[2M+Na-H]^+$, 50%.

4.2.5. Dimer *N*-Fmoc-L-T-T-OBn (7). *Compound* **4a** (0.31 g, 0.5 mmol) was suspended in dry DCM (2 mL) and then *N*,*N*-diethylamine (1 mL) was added at room temperature. After reacting for 30 min, the solvent was evaporated in vacuo to give the crude amine-free chiral monomer **6**. It was dissolved in dry DMF (2 mL) and stored at −10°C and directly used in the next step of coulping without purification. L-monomer **5a** (0.260 g, 0.5 mmol), FEP (0.14 g, 0.55 mmol) and DIPEA (360 μL, 1.25 mmol) were dissolved in dry DMF (2 mL) and dry DCM (2 mL), and then cooled to −10°C in an ice–salt bath. The DMF solution of **6** was added to this stirred mixture at low temperature. The reaction was continued for 2 h at the same temperature, and an additional 3 h at room temperature under stirring. The mixture was concentrated in vacuo and the residue was purified by flash chromatography (ethyl acetate–methanol–water 25:1:0.1) to give the dimer as a white foam (0.25 g, 56%). [Found: C, 61.55; H, 5.73; N, 12.26%. $C_{46}H_{50}N_8O_{11}\cdot 1/2H_2O$ requires C, 61.39; H, 5.71; N, 12.45%]; ν_{max}(KBr): 1681 (C=O); 759, 739 (Fmoc and -Ph) cm^{-1}; ^1H NMR (400 MHz, d_6-DMSO) more than two isomers δ 7.90–7.31 (15H, m, phenyl and Fmoc-aromatic-C*H* and T-C*H*(6)), 5.20 and 5.11 (2H, 2×s, CH_2Ph), 4.68–4.02 (9H, m, C*H*Me, Fmoc-aliphatic-C*H* and –CH_2 and thymine-CH_2CO,), 3.8–3.0 (8H, m, H_2O and –CH_2NCH_2), 1.73–1.64 (6H, brm, thymine-CH_3), 1.18–0.97 (6H, 2×m, CH_3); ESIMS *m/z*: 891.5 $[M+H]^+$, 100%, 913 $[M+Na]^+$, 75%, 669.4 $[M-Fmoc-CO_2+H]^+$, 20%.

4.2.6. *N*-Fmoc-L-T-T-OH (8). The synthetic procedure was similar to that of *N*-Fmoc-L,D-T-OH, white solid. ν_{max}(KBr): 3303 (OH), 1681 (C=O), 761, 739 (Fmoc) cm^{-1}; ^1H NMR (400 MHz, d_6-DMSO) more than two isomers δ 11.24 (1H, brm, COO*H*), 7.89–7.16 (10H, m, Fmoc aromatic-C*H* and T-C*H*(6)), 4.82–3.6 (13H, m, C*H*Me, Fmoc aliphatic-C*H* and –CH_2 and thymine-CH_2CO, NCH_2CO), 3.55–3.0 (4H, m, H_2O and CHCH_2N), 1.68 (6H, brm, T-CH_3), 1.23–1.00 (6H, 2×m, CHCH_3); ESI-MS: 803 $[M+2H]^+$, 100%, 846 $[M+2Na-H]^+$, 90%, 867.3 $[M+3Na-2H]^+$, 45%.

4.2.7. *N*-(Fluoren-9-ylmethoxycarbonyl)amino-penta-1-yl succinate (10). Compound **9** (0.81 g, 2.5 mmol) was dissolved in dry DCM (10 mL) and dry pyridine (1 mL). To this solution was added succinic anhydride (0.25 g, 2.5 mmol) and DMAP (30.4 mg, 0.25 mmol). After stirring for 3 h at room temperature, an additional portion of succinic anhydride (25.0 mg, 0.25 mmol) and DMPA (60.8 mg, 0.50 mmol) were added and the solution was stirred for 6 h at 50°C and 16 h at room temperature. The solution was evaporated in vacuo, the residue was dissolved in ethyl acetate and washed once with ice cold 5% citric acid. The organic phase was dried under Na_2SO_4, filtered and evaporated under reduced pressure. The residue was purified by flash chromatography (from DCM–ethyl acetate 2:1 to DCM–methanol 50:1) to give the product **11** as a white solid (0.59 g, 65%). [Found: C, 67.99; H, 6.40; N, 3.21%. $C_{24}H_{27}NO_6$ requires C, 67.75; H, 6.40%; N, 3.29%]; ν_{max}(KBr): 3352 (OH), 1716, 1689 (C═O), 758, 738 (Fmoc) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 7.78–7.26 (8H, m, Fmoc-aromatic-C*H*), 4.97 (1H, s, FmocN*H*), 4.47–4.40 (2H, m, $-CH_2O$), 4.23–4.09 (3H, m, Fmoc-aliphatic-C*H* and $-CH_2$), 3.17 (2H, m, NCH_2), 2.64 (4H, m, $COCH_2CH_2CO$), 1.66–1.37 (6H, m, $CH_2CH_2CH_2CH_2CH_2$); ESIMS *m/z*: 448 $[M+Na]^+$, 100%, 873 $[2M+Na]^+$, 80%.

4.3. *N*-(Fluoren-9-ylmethoxycarbonyl)amino-penta-1-yl succinylamido-BHA resin

BHA resin (100–200 A, 0.539 mmol g^{-1}, 0.200 g) was placed in a manually operated solid-phase reaction vessel and treated with 10% NEM in DCM for (2×15 min) and washed with DCM. A solution of **10** (107 mg, 0.25 mmol), DIPEA (65 μL, 0.375 mmol) and AOP (111 mg, 0.25 mmol) in DCM was immediately added to BHA resin. The resulting suspension was shaken for 3 h at room temperature. The derivatized BHA resin was filtered, washed with DMF (3×2 mL), EtOH (3×2 mL), DCM (3×2 mL) successively, and dried in vacuo.

4.4. Measurement of loading

The loading of the supports was determined by the spectrophotometric (301 nm) determination of the concentration of dibenzofulvene–piperidine obtained by deprotection of Fmoc group from a sample of support. The extinction coefficient of the dibenzofulvene–piperidine (ξ_{264} 18,000 $dm^3\ mol^{-1}\ cm^{-1}$) was used for this calculation. The loading was calculated to be 0.436 mmol g^{-1}.

4.5. Stepwise synthesis of 10mer chiral PNAs

The synthesis was started from 87 mg (dry weigh) of *N*-5-Fmoc-amino-penta-1-yl-succinylamido-BHA resin, which was pre-swelled in DCM overnight. The procedure is as follows: (1) Fmoc deprotection with 20% piperidine in DMF, 2×15 min; (2) washing with DMF (3×2 mL), EtOH (3×2 mL), DCM (3×2 mL); (3) coupling using a 0.05 M of solution of Fmoc-amino acid **5a** or **5b** in DMAc–DCM (1:2) containing AOP (0.05 M), HOAt (0.05 M) and DIPEA (0.075 M). The mixture was allowed to proceed for 3 h at room temperature; (4) washing with DMAc (3×2 mL), EtOH (3×2 mL), DCM (3×2 mL); (5) capping of the unreacted amino group using 5% Ac_2O in DMF, 2×15 min; (6) washing with DMAc (2×2 mL), EtOH (2×2 mL), DCM (2×2 mL);. Steps 1–6 were repeated until the desired sequence were obtained. After the final coupling, the resins were treated with 0.5 M NaOH in water–THF (1:1) for 3 h at room temperature. Then, the solution was collected and adjusted to pH 7.0 by aq. 1 M AcOH. After concentration to dryness, the residue (ESI-MS of crude PNA *m/z* 2906.0) was dissolved in H_2O and purified on kromasil RP-C18 (4.6×250 mm), eluting with a linear gradient of 20–23% B in A (A: 0.05% TFA in water; B: 0.05% TFA in acetontrile) yielding 62% of oligmer H_2N-$(T)_{10}$-(penta).

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