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Dde-protected PNA monomers, orthogonal to Fmoc, for the synthesis of PNA–peptide conjugates

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Abstract—Peptide nucleic acids have become, arguably, one of the most interesting of DNA mimics. Herein the efficient solution phase synthesis of four novel 1-(4,4-dimethyl-2,6-dioxacyclohexylidene)ethyl/4-methoxytrityl (Dde/Mmt) protected PNA monomers is reported which were then used to synthesise PNA–peptide conjugates through a mild Dde deprotection strategy, which was fully orthogonal to Fmoc chemistry, allowing at will Fmoc peptide and Dde–PNA synthesis.

1. Introduction

Peptide Nucleic Acids (PNAs) were first reported in 1991 as a DNA mimic[1] and since this time, a vast number of studies have been reported covering their synthesis, properties and potential applications.[2] In PNAs, the sugar–phosphate backbone present in DNA has been replaced by a repeating polyamide chain of *N*-(2-aminoethyl)glycine monomer units, yet despite this major modification PNA still hybridises efficiently to complementary DNA and RNA sequences according standard base pairing rules (Fig. 1); indeed, due to the lack of electrostatic repulsion in PNA/DNA complexes present in DNA/DNA or DNA/RNA double strands, the binding affinity and selectivity of PNA towards DNA, RNA and PNA is higher than for its DNA counterparts under physiological conditions. Moreover, PNA is resistant to biological degradation by nucleases or proteases, properties which make PNA an ideal tool in a

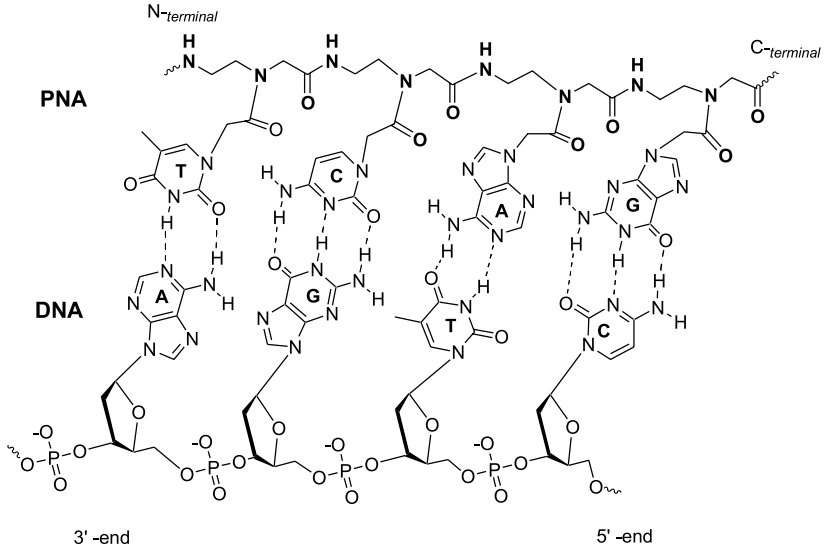

Figure 1. General structure of a PNA/DNA duplex.

Keywords: PNA; Dde; Fmoc; Orthogonality; Conjugate; Peptide.
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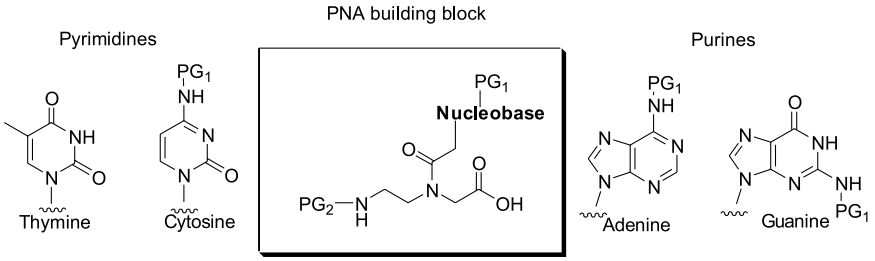


Figure 2. General structure of the four PNA building blocks.

number of different areas of research such as gene therapy, SNP analysis and mRNA profiling.[3] A further advantage of PNA over other DNA mimetics stems from its pseudo-peptidic structure which allows PNA synthesis to be carried out by straightforward and traditional solid phase peptide chemistry based methods (SPPS). However PNA does suffer from a number of drawbacks and disadvantages, such as issues relating to its cellular delivery and uptake, the inability to enzymatically extend PNA primers and the complexities of PNA synthesis, which to date has made concurrent peptide/PNA synthesis and PNA/DNA synthesis very inefficient. However, the conjugation of PNA to peptides can be used to enable the properties of the PNA to be highly modulated, for example, by allowing membrane permeability which then opens up a host of opportunities for new applications of PNA and better exploitation of its full potential. Therefore, methods to allow the efficient preparation of peptide–PNA conjugates in which the peptide and PNA strands can be prepared at will would have huge potential.[4]

To allow the construction of peptide–PNA conjugates, monomeric building blocks protected with two orthogonal protecting groups (one for the N terminus and one for the nucleobase) are required, and during the past decade PNA monomers with a range of compatible protecting groups have been developed with Fmoc/Bhoc[5] and Boc/Cbz[6] being the most frequently used but other combinations such as Fmoc/Mmt[7] or Fmoc/Cbz[8] have also been used. While these monomers are useful for the synthesis of standard PNA oligomers, their utility in the synthesis of PNA–peptide conjugates is limited as they do not allow orthogonal synthesis with commercially available amino acid monomers (the most frequently used being the N-terminal Fmoc protected amino acids with acid labile side-chain protecting groups).

In the quest for alternative N-terminal protecting groups for PNA monomers which would allow the desired degree of orthogonality, 1-(4,4-dimethyl-2,6-dioxacyclohexylidene) ethyl (Dde) was identified, as a protecting group which has been used for primary amines and one that is classically removed with hydrazine.

Here, we describe the synthesis of novel Dde/Mmt PNA monomers and the development of new deprotection conditions for the Dde group which were robust and fully orthogonal to other commonly used protecting groups in peptide chemistry, such as Fmoc, Boc, Mmt, ^{t}Bu, MeO, etc. We further demonstrate the application of these monomers to a highly versatile synthesis of various PNA–peptide conjugates.

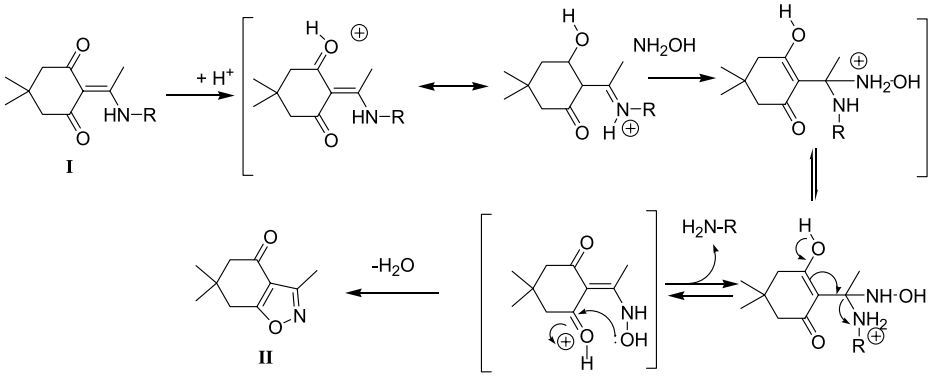
2. Results and discussion

In order to carry out solid phase PNA oligomers synthesis, PNA monomeric building blocks protected on the amino group of the *N*-(2-aminoethyl)glycine backbone as well as the nucleobases (Fig. 2), had to be prepared.

It was desired that the PNA and the peptide moieties should be elongated in a truly orthogonal fashion, thus imposing no restrictions on the design of these conjugates, but protecting groups on the nucleobases (PG_1) and the amino acids should be cleaved together under the final acidic cleavage conditions. The use of an acid labile linker such as the Rink amide linker[9] would also enable deprotection to be performed during the final cleavage of the conjugate from the solid-support. For this purpose, the mono-methoxytrityl (Mmt) group was chosen for the nucleobase protecting group (PG_1). The correct choice of PG_2-which had to be orthogonal to the Fmoc group and the acid-labile side-chain protecting groups on peptides proved more challenging, as none of the PNA monomers reported to date met these criteria.

As a possible solution, the allyloxycarbonyl (Aloc) group, which can be cleaved by palladium catalysts under essentially neutral conditions,[10] was examined. Although the Aloc group can be deprotected without affecting the Fmoc group, the use of Aloc-protected PNA monomers, synthesized in our group, proved to be unsuccessful in chain extension with the expected products obtained in very low yield and purities. Moreover, the length of the PNA oligomers was severely affected as during Aloc deprotection, Pd(0) species become trapped within the solid supports (generating black beads) causing premature and random Aloc deprotection. Thus synthesis on solid support using Aloc protected monomers in a coupling–deprotection strategy was fundamentally flawed.

Hence, after unsuccessful attempts to use Aloc-PNA monomers for the oligomerization process, another protecting group was identified, 1-(4,4-dimethyl-2,6-dioxacyclohexylidene)ethyl (Dde). While hydrazine, the usual reagent to deprotect the Dde group, also deprotects the Fmoc group, we reasoned that the Dde group should be deprotected by hydroxylamine (mixture of $NH_2OH{\cdot}HCl$ and imidazole) under slightly acidic conditions which would be fully orthogonal to the Fmoc group.[11] The principle of the orthogonality being based on the different mechanisms involved for both deprotections. While Fmoc protected amines are deprotected through an E1cb elimination generating dibenzofulvene, the Dde group is cleaved by



Scheme 1. Possible Dde deprotection mechanism using $NH_2OH \cdot HCl$/imidazole.

nucleophiles through a transenamination or Michael type mechanism followed by ring closure yielding an isoxazole **II** (Scheme 1, Fig. 3).

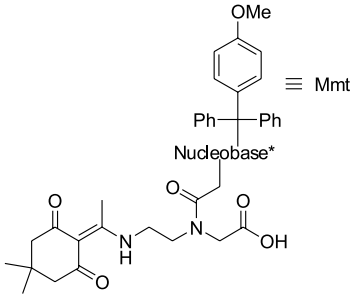


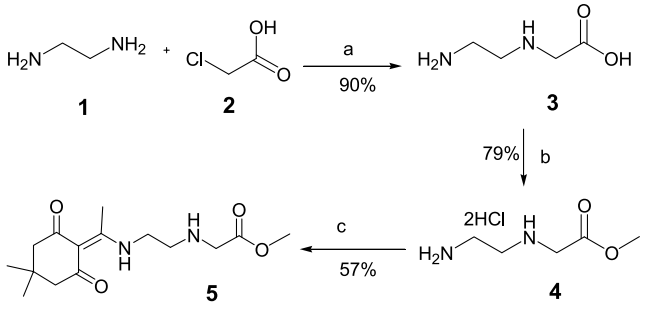
Figure 3. Novel Dde/Mmt PNA monomers. *Thymidine monomers do not require nucleobase protection.

2.1. Synthesis of Dde/Mmt PNA monomers

The synthesis of the PNA monomers containing the four nucleobases was carried out in solution. Retro-synthetically, the monomers can be disconnected into two synthons, a backbone and the derivatised nucleobase and this allows the straightforward synthesis of all four desired PNA monomers.

2.2. Synthesis of the Dde-protected backbone

The synthesis of the Dde-protected backbone **5** is depicted in Scheme 2. Thus, chloroacetic acid was alkylated with an excess of ethylendiamine **1**, which following precipitation with DMSO gave 2-aminoethylglycine **3** in quantitative yield.[12] Following esterification of **3**, Dde protection was carried out using Dde-OH previously prepared from dimedone and acetic acid,[13] yielding the PNA backbone ethyl 2-*N*-Dde-aminoethylglycinate **5** in an overall yield of 40% (Scheme 2).

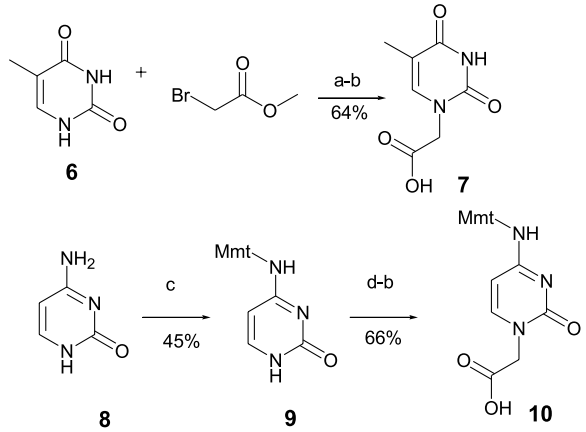


Scheme 2. Dde backbone synthesis: (a) room temperature, 16 h; (b) 10 equiv SO_2Cl, MeOH, reflux, 16 h.; (c) 2 equiv DIPEA, 1 equiv Dde-OH; CH_2Cl_2/EtOH (1/1), room temperature, 16 h.

2.3. Derivatization of the nucleobases

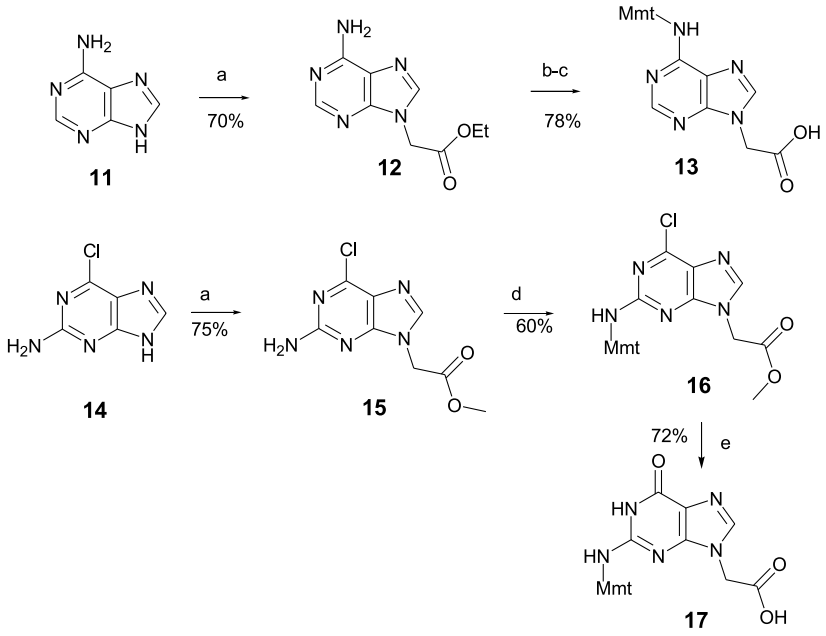
The nucleobases were derivatized through alkylation and protection of the exocyclic amino group, if necessary, with the monomethoxytritryl group (Mmt) following a procedure reported by Breihpol.[7]

Thymine **6** was thus alkylated following the procedure by Buchardt,[6] by treatment of commercially available thymine with methyl bromoacetate using K_2CO_3 as base, followed by saponification of the resulting compound with NaOH yielding thymin-1-yl acetic acid **7**. Cytosine, was first protected with Mmt-Cl in pyridine and then alkylated using a similar procedure as described for **7** (Scheme 3).



Scheme 3. Pyrimidine nucleobases derivatizion: (a) 1 equiv K_2CO_3, DMF under N_2, 16 h; (b) 10% NaOH (aq), reflux, 10 min; (c) 1.5 equiv Mmt-Cl, 1 equiv NEM, Pyr, 40 °C, overnight; (d) 1 equiv K_2CO_3, 1 equiv methyl 2-bromoacetate, DMF, under N_2, 16 h.

In order to obtain selective N^9 over N^7 alkylation, purine nucleobases were first alkylated followed by exocyclic amino protection. Following deprotonation of adenine with sodium hydride, alkylation was carried out using ethyl bromoacetate to give **12**. Amino protection using Mmt chloride in pyridine and subsequent saponification yielded [N^6-(Mmt)-adenin-9yl]-acetic acid **13** (Scheme 4).



Scheme 4. Purine nucleobases derivatization: (a) 1.1 equiv NaH, 1 equiv methyl 2-bromoacetate, DMF, under N_2, 4 h; (b) 1.5 equiv Mmt-Cl, 1 equiv NEM, CH_2Cl_2, 40 °C, 3 h, then 25 °C, 16 h; (c) 1 N aqueous NaOH, reflux, 2 h; (d) 1.5 equiv Mmt-Cl, 1 equiv DIPEA, THF, overnight; (e) 10% NaOH (aq.), reflux, 2 h.

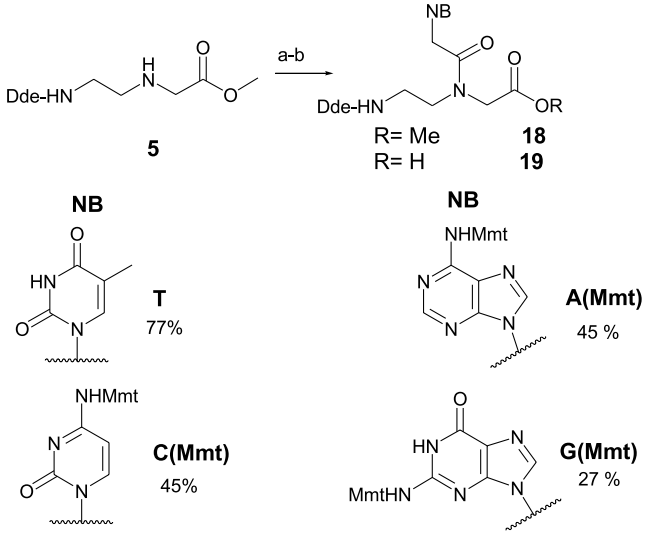
2-Amino-6-chloropurine **14** was used as precursor of the guanine nucleobase. This was due to the poor solubility of guanine, the lack of reactivity of its exocyclic amino group and the difficulty in obtaining selectively the N^9 alkyl derivatives. Alkylation and Mmt protection were carried out as for adenine, however dry CH_2Cl_2 instead of pyridine was used in the Mmt protection step. The use of pyridine as solvent led to the formation of the 6-pyridinium derivative as a side product. In the last step, basic hydrolysis of the ester moiety as well as the C-6 chlorine substituent provided [N^2-(Mmt)-guanin-9-yl]-acetic acid **16** (Scheme 4).

2.4. Monomer synthesis

Two steps remained for the synthesis of the monomers: coupling of the protected backbone **5** and the derivatised nucleobases **7**, **10**, **13**, and **16**, and hydrolysis of the ester moiety. Amide formation with the secondary amine moiety in **5** proved to be sluggish. Protected nucleobases-acetic acid derivatives, **10**, **13** and **17** (mainly purine), are known to be difficult substrates for amide formation and therefore various different coupling reagents were screened.

Couplings of pyrimidine nucleobase derivatives **7** and **10** to the backbone **5** were achieved using different coupling reagents such as 1-propylphosphonic acid cyclic,[14] PyBroP and DCC/DhbtOH[6] with similar efficiencies. Esters **18T** and **18C**(**Mmt**) were obtained with average yields of 60%. PyBroP proved to be the most efficient coupling agent (according to HPLC analysis) with complete conversion however, with this coupling reagent, the purification process was challenging, especially on a large scale. In fact removal of *tris*-(pyrrolidin-1-yl)-phosphoramide was best performed after ester hydrolysis by repeated precipitation (see Section 4).

The second step involved basic hydrolysis of the ester group. Among the conditions tested (LiOH in THF, LiOH in MeOH/H_2O, NaOH in MeOH/H_2O, KOH in H_2O, Na_2CO_3 in MeOH/H_2O, K_2CO_3 in MeOH/H_2O), a 1 M solution of cesium carbonate in MeOH/H_2O (1:1) gave the best results (**19a**–**d**). The lack of Dde deprotection during saponification is a major advantage compared to the preparation of Fmoc protected PNA monomers, where an extra Fmoc re-protection step is often required following ester hydrolysis.[7] Another major advantage of the synthesis of Dde-protected monomers is that no chromatographic separations were needed, with only extraction and precipitation procedures used. The final products were obtained as pure materials (as determined by HPLC and NMR), allowing multi-gram quantities of the monomers to be



Scheme 5. Dde protected PNA monomers: (a) T-CH_2COOH, 50% 1-propylphosphonic acid cyclic in DMF, NEM, 16 h (**18T**); C(Mmt)CH_2-COOH or A(Mmt)CH_2COOH or G(Mmt)CH_2COOH, PyBroP, DIPEA, DMF, 2.5–15 h (**18C**(**Mmt**)-**18A**(**Mmt**)–**18G**(**Mmt**)); (b) 1 M Cs_2CO_3 MeOH/H_2O 1/1, 1.5 h. NB = Nucleobases.

easily synthesized following our protocols, indeed we have prepared up to 5 g of monomers using this synthetic route (Scheme 5).

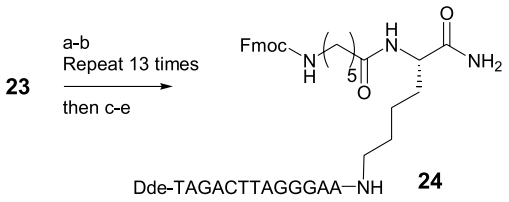
2.5. PNA–peptide conjugates

The Dde PNA monomers open up new opportunities for the flexible synthesis of PNA conjugates. In a previous communication, our group reported the orthogonality between Dde and Fmoc protecting groups.[11] Furthermore the Dde deprotection protocol was completely compatible with the Mmt protecting group and the nucleobases. To demonstrate this robustness we embarked on the synthesis of various different conjugates.

Fmoc-Lys(Dde)-OH **21** was used as core of these conjugates (Scheme 6)[15] which was coupled to commercially available Rink amino PEGA resin, using PyBOP, to give **22**. Three possible strategies are realizable for the construction of PNA–peptide conjugates: (a) building up first the PNA arm (using Dde monomers **19**), and then the peptide arm (using Fmoc monomers); (b) or vice versa; (c) or alternating PNA elongation and peptide elongation steps.

The synthesis of the PNA oligo arm was firstly investigated. Initially three different resin types were tested: PS, Tentagel and PEGA but in terms of Dde deprotection, PEGA showed the fastest cleavage kinetics (complete deprotection after 1 h); while the other resins required longer times (up to 3 h).[11] PEGA was thus considered most suited for the purpose of PNA synthesis with Dde PNA monomers and after selective Dde deprotection with a solution of $NH_2OH \cdot HCl$/imidazol (1/0.75 equiv) in NMP/DMF (5/1) for one hour resin **23** (as the salt) was obtained.

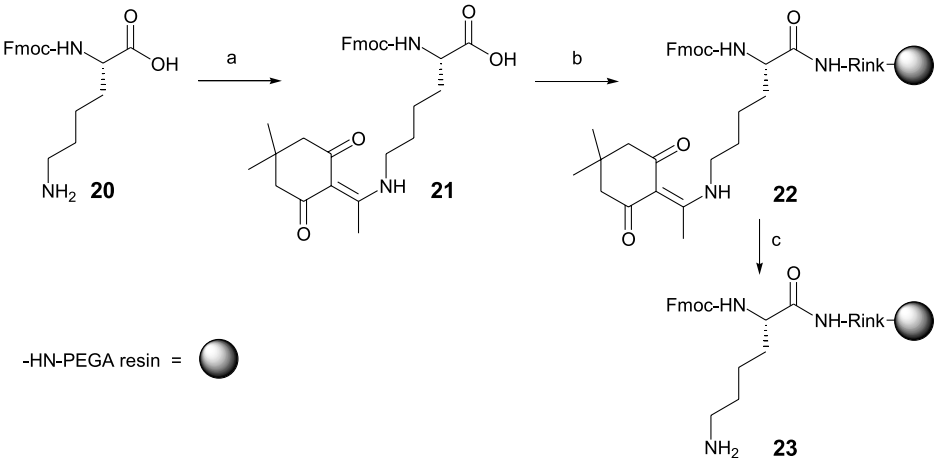
A 13mer PNA oligo containing the four natural nucleobases was synthesised starting from **23** (Scheme 7) with PNA monomer couplings achieved with PyBOP/NEM in DMF using 5.5 equiv of monomer (0.11 M) for 3 h while Dde deprotection was achieved using the protocol described above. After elaboration of the PNA arm, Fmoc deprotection was carried using 20% piperidine in DMF and after coupling of Fmoc–aminohexanoic acid the PNA intermediate **24** was cleaved with a mixture of TFA/TIS/CH_2Cl_2 (90/5/5) for 1 h. After precipitation from TFA–diethyl ether, the purity and identity of the crude material was determined by HPLC and MALDI-TOF, where no loss of the Fmoc group could be detected. Thus, the new monomers were demonstrated to be valid building blocks for PNA oligomerization.



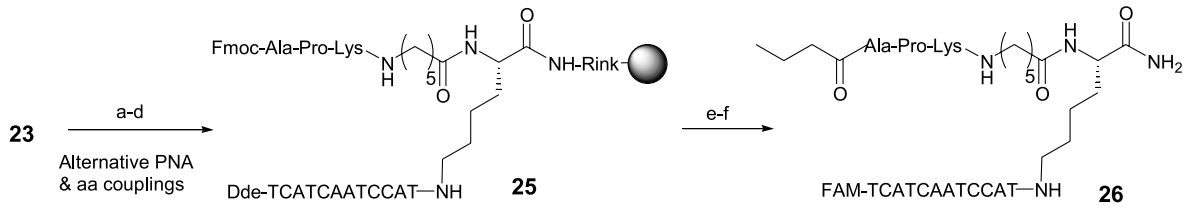
Scheme 7. Synthesis of a 13 mer PNA oligo using Dde protected PNA monomers: (a) Dde-PNA-OH (5.5 equiv), PyBOP (5 equiv 0.1 M), NEM (11 equiv) in DMF, 3 h; (b) $NH_2OH \cdot HCl$/imidazole in NMP/DMF; repeat (a) and (b) as necessary; (c) excess 20% piperidine in DMF; (d) Fmoc-Ahx-OH (5.5 equiv), PyBOP (5 equiv, 0.1 M), DIPEA (11 equiv) in DMF, 3 h; (e) excess TFA/TIS/CH_2Cl_2 (90/5/5), 1 h.

Traditionally, peptides have been conjugated to PNA using either traditional solid phase chemistry by coupling amino acids followed by PNA monomers or vice versa[16–18] using disulfide linkage[19] or ligation through disulfide bonds.[20] However, the method reported here allows a much more flexible strategy which involves elongation of either PNA or peptide parts at any stage of the synthesis (see above). To demonstrate this flexibility, a conjugate was synthesised by alternating PNA coupling and amino acids coupling steps (Scheme 8). PNA monomer couplings were performed as for compound **24** and amino acid couplings were carried out using PyBOP/DIPEA in DMF with HOBt for 3 h. Fmoc deprotection was carried out using 20% piperidine in DMF for two cycles of 6 min. No Dde cleavage was observed under these conditions.

Once the construct **25** was prepared the peptide arm was capped using butyric acid and the conjugate was labelled with 5(6)-carboxyfluorescein on the PNA arm, to yield **26**. Following cleavage from the resin with TFA/TIS/CH_2Cl_2



Scheme 6. Fmoc-Lys-Rink-PEGA 23 synthesis: (a) Dde-OH (2 equiv, 26 mM), TFA (0.1 equiv) in EtOH, reflux, 2 days;[15] (b) **21** (5.5 equiv), PyBOP (5 equiv, 0.1 M), amino-Rink-PEGA resin, DIPEA (11 equiv) in DMF, 3 h; (c) 20% $NH_2OH.HCl$/imidazole (1/0.75 equiv) in NMP/DMF (5/1).



Scheme 8. Synthesis of **26** using alternative PNA and amino acid couplings: (a) Dde-PNA-OH (5.5 equiv), PyBOP (5 equiv), NEM (11 equiv) in DMF, 3 h; (b) 20% piperidine in DMF; (c) Fmoc-AA-OH or butyric acid (last step) (5.5 equiv), PyBOP (5 equiv, 0.08 M), DIPEA (16 equiv), HOBt (5.5 equiv) in DMF, 3 h; (d) $NH_2OH.HCl$/imidazole; repeat a–d as necessary; (e) 5(6)-carboxyfluorescein (22 equiv), PyBOP (22 equiv, 0.08 M), NEM (44 equiv), in DMF, 15 h; (f) TFA/TIS/CH_2Cl_2 (90/5/5), 1 h. FAM = 5(6)-carboxyfluorescein.

(90/5/5) and precipitation, HPLC and MALDI-TOF analysis was undertaken to give the data shown in Figure 4.

3. Conclusion

The solution phase synthesis of a new class of PNA monomers and their application to PNA oligomerization is presented. These monomers present an alternative to Fmoc protected PNA monomers, and the Dde monomers show distinct advantages, especially in the context of PNA–peptide conjugates giving the possibility of a more flexible strategy to obtain PNA–peptide conjugates, such as the possibility of a truly orthogonal synthesis of PNA and peptide when using commercially available Fmoc amino acids. This strategy is routinely used in our group for the synthesis of split and mix PNA encoded peptide libraries which require alternative coupling steps for amino acid and PNA monomers. Further possible applications beyond the synthesis of PNA–peptide conjugates include the synthesis of PNA molecular beacons (i.e., the complementary PNA arm constructed with Fmoc protected PNA monomers). However, it should be noted that PNA poly-T sequences (more than four Ts in a row) cannot be synthesized with this method, due to the strong interactions of polythymidine chains within the beads.

4. Experimental

4.1. General

NMR spectra were recorded using a Bruker AC300 spectrometer operating at 300 MHz for ^1H and 75 MHz

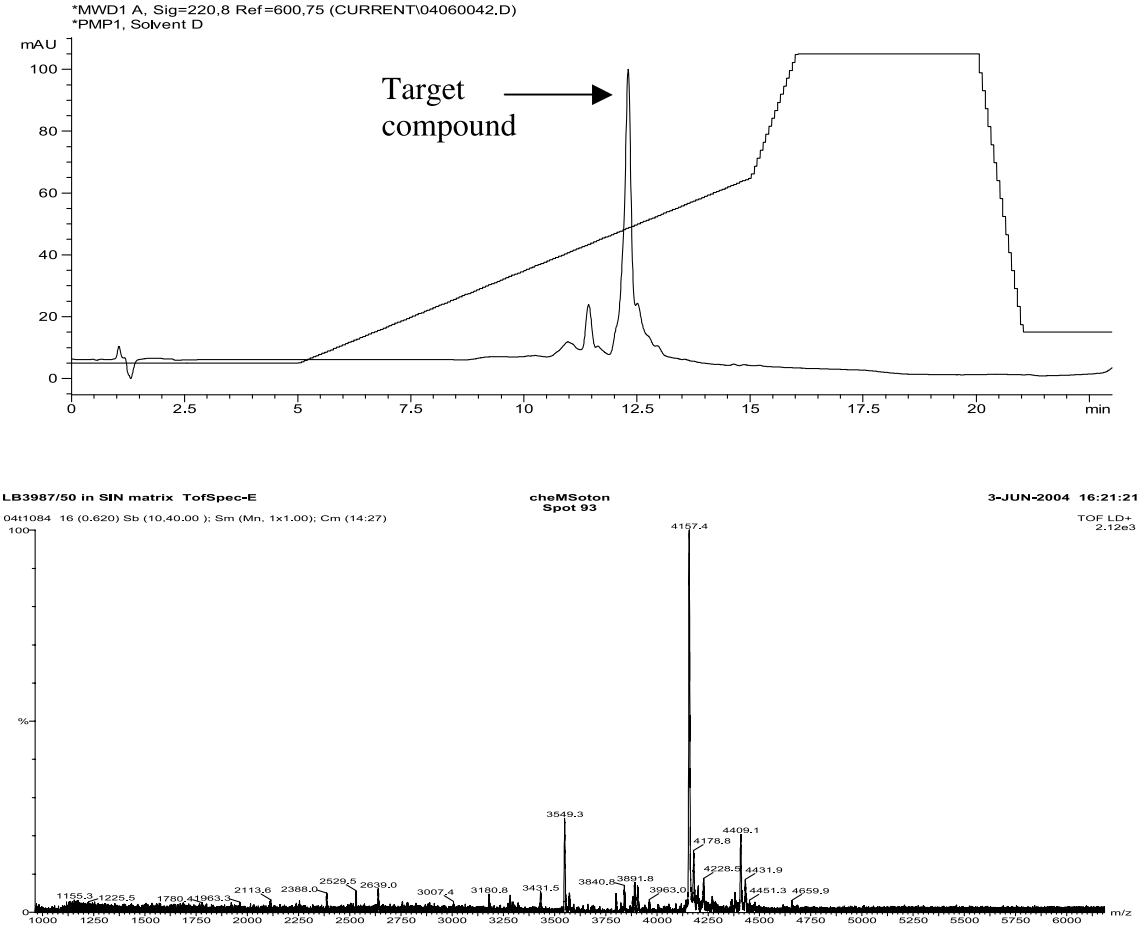


Figure 4. HPLC (acetonitrile gradient is shown, $\lambda = 220$ nm) and MALDI-TOF of crude compound **26**.

for ^{13}C and a Bruker AC400 spectrometer operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C. All coupling constants (J values) were measured in Hz. ES mass spectra were recorded using a VG Platform Quadrupole Electrospray Ionisation mass spectrometer. Reverse phase analytical HPLC (RP HPLC) was performed using a Hewlett Packard HP1100 Chemstation, using a Phenomenex C_{18} prodigy 5 μm (150 mm×3.0 mm i.d.) column. The following methods were used: solvent A=H_2O/CH_3CN/TFA 90/10/0.1 to solvent B=H_2O/CH_3CN/TFA 10/90/0.1, solvent C=H_2O/CH_3CN/TFA 100/0/0.1, solvent D=H_2O/CH_3CN/TFA 0/100/0.1.

Method 1 (flow rate 0.5 mL/min). 100% A to 100% B over 10 min, followed by 100% B for 5 min. Method 2 (flow rate 1 mL/min) 100% A H_2O to 100% B over 3 min followed by 100% B for 1 min. Method 3 (flow rate 0.5 mL/min); 100% C over 5 min, then 100% C to 40% C/60% D over 10 min then to 100% D followed by 100% D over 4 min).

Retention time (R_t). Thin layer chromatography (TLC) was performed using Alugram SIL G/UV/254 precoated plates. Visualisation was achieved by UV radiation.

PyBOP (benzotriazole-1-yl-oxy-tris-pyrrolidinio-phosphonium hexafluorophosphate) and PyBroP (bromo-tris-pyrrolidinio-phosphonium hexafluorophophate) were purchased from Novabiochem (Merck Bioscience, UK). PEGA resin (acrylolated *O*,*O*-bis(aminoproplyl)polyethylene glycol resin) 0.4 mmol/g 150–300 μm was purchased from Polymer Labs (UK), DIPEA (*N*,*N*-di*iso*propyl-*N*-ethylamine), NEM (*N*-ethylmorpholine).

4.1.1. Methyl *N*-{2-[1-(4,4-dimethyl-2,6-dioxo-cyclohexyliden)-ethylamino]-ethyl}-glycinate (5). To a stirred solution of diamine salt **4**[12] (41.6 g, 203 mmol) and DIPEA (71 mL, 406 mmol) in CH_2Cl_2/MeOH (1/1 500 mL) was added Dde-OH (37 g, 203 mmol). The solution was stirred at room temperature for 16 h before evaporating the solvent in vacuo. The crude material was taken up in EtOAc (400 mL) and extracted with 1 M $KHSO_4$ (4×100 mL), which was brought to pH 9 with $NaHCO_3$ before extraction with further EtOAc (4×100 mL). The organic phases were combined, washed with brine (1×100 mL), dried over $MgSO_4$ and concentrated in vacuo to give ester **5** as a brown oil. (34.2 g, 57%). R_f=0.34 (EtOAc); HPLC (method 2) R_t=2.5 min; mp 37 °C; HRMS (ES+): m/z calcd for $C_{15}H_{24}N_2O_4$ [M+H^+] 297.1808, found 297.1807; ^{1}H NMR (400 MHz, $CDCl_3$): δ=13.42 (s, 1H, NH-Dde), 3.66 (s, 3H, OCH_3), 3.43–3.48 (m, 4H, CH_2), 2.87 (t, ^{3}J(H,H)=6.2 Hz, 2H, CH_2), 2.50 (s, 3H, CCH_3), 2.29 (s, 4H, CH_2-Dde), 1.96 (s, 1H, NH), 0.95 (s, 6H, (CH_3-Dde) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ=198.1 (CO-Dde), 173.4 (*C*CH_3), 172.7 (*C*$OOCH_3$), 107.9 (Dde-*C*=C), 52.8 (CH_2-Dde), 51.8 (OCH_3), 50.2 (NH*C*H_2CO), 47.6 (DdeNHCH_2*C*H_2NH), 44.0 (DdeNH*C*H_2CH_2NH), 30.0 ((CH_2)$_2$*C*(CH_3)$_2$), 28.2 (CH_3-Dde), 18.1 (C–*C*H_3).

4.1.2. [N^4-(4-Methoxytrityl)-cytosin-1-yl]-acetic acid (10). NaH (1.04 g, 60% in mineral oil, 26 mmol) was added to a stirred suspension of pyrimidine **9**[7] (10 g, 26 mmol) in DMF (100 mL). After stirring for 1 h at room temperature the mixture was cooled to 0 °C and methyl 2-bromoacetate (3.43 mL, 31.3 mmol) was added dropwise. The reaction was stirred at room temperature for 16 h before evaporating the solvent in vacuo. The resulting oil was precipitated with H_2O and removed by filtration, and washed with H_2O to give a white solid which was dried over P_2O_5 in vacuo. R_f=0.32 (EtOAc); HPLC (method 2) R_t=3.43; mp>200 °C min; HRMS (ES+): m/z calcd for $C_{27}H_{25}NaN_3O_4$ [M+Na^+] 478.1737, found 478.1741; ^{1}H NMR (300 MHz, d_6-DMSO): δ=12.8 (br s, 1H, COOH), 8.4 (br s, 1H, NH), 7.45 (d, ^{3}J(H,H)=7.7 Hz, 1H, CH_{Cyt}) 7.4–7.1 (m, 12H, CH_{Mmt}), 6.81 (d, ^{3}J(H,H)=8.8 Hz, 2H, CH_{Mmt}), 6.18 (d, ^{3}J(H,H)=7.7 Hz, 1H, CH_{Cyt}), 4.25 (s, 2H, CH_2), 3.70 (s, 3H, H_3CO–Ar), 3.60 (s, 3H, $COOCH_3$) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ=168.4 (COOH), 166.0 (=C_{Cyt}), 158.8 (*C*OCH_3), 155.7 (CO), 145.2 (CH_{Mmt}), 144.2 (C_{Mmt}), 135.9 (C_{Mmt}), 130.0 (CH_{Cyt}), 129.0 (CH_{Mmt}), 128.6 (CH_{Mmt}), 127.8 (CH_{Mmt}), 113.6 (CH_{Mmt}), 94.9 (CH_{Cyt}), 70.6 (C_{Mmt}), 55.2 (OCH_3), 52.6 ($COOCH_3$), 50.1 (CH_2).

Methyl [N^4-(4-methoxytrityl)-cytosin-1-yl]-acetate[7,21] (31.1 g, 67 mmol) was suspended in 2 N aqueous NaOH (200 mL) and the mixture was stirred for 2 h at reflux. The reaction was cooled to room temperature, washed with CH_2Cl_2 (3×50 mL) and acidified with 2 N aqueous HCl until pH 2. The precipitated was collected by filtration, washed with small portions of water until the filtrate was neutral (pH=7) and dried in vacuo for 12 h to give acid **10** (11.4 g, 66% over two steps) as a white solid.; R_f=HPLC (method 2); R_t=3.33 min; mp 210 °C; HRMS (ES^+) m/z calcd for $C_{26}H_{24}N_3O_4$ [M+H^+] 442.1762; found 442.1770; ^{1}H NMR (400 MHz, d_6-DMSO): 8.31 (s, 1H, NH_{Mmt}), 7.44 (d, ^{3}J(H,H)=6.5 Hz, 1H, CH_{Cyt}), 7.27–7.16 (m, 12H, CH_{Mmt}), 6.82 (d, ^{3}J(H,H)=5.5 Hz, 2H, C*H*$COCH_3$), 6.17 (d, ^{3}J(H,H)=5.8 Hz, 1H, CH_{Cyt}), 4.24 (s, 2H, CH_2), 3.72 (s, 3H, H_3CO–Ar) ppm; ^{13}C NMR (100 MHz, d_6-DMSO): 169.9 (COOH), 164.1 (C_{Cyt}), 158.6 (*C*–OCH_3), 157.5 (CO), 144.9 (CH_{Mmt}), 144.2 (C_{Mmt}), 135.1 (C_{Mmt}), 129.9 (CH_{Cyt}), 128.6 (CH_{Cyt}), 127.4 (CH_{Cyt}), 126.1 (CH_{Mmt}), 112.8 (CH_{Mmt}), 95.5 (CH_{Cyt}), 69.7 ($C(Ar)_3$), 55.0 (O–CH_3), 49.7 (*C*H_2COOH) ppm.

4.1.3. [N^6-(4-Methoxytrityl)-adenin-9-yl]-acetic acid (13). A mixture of ester **12**[6] (7.21 g, 32.3 mmol), NEM (4.1 mL, 32.3 mmol) and 4-monomethoxytrityl chloride (15.0 g, 48.5 mmol) in pyridine (100 mL) and dichloromethane (100 mL) was heated at 40 °C for 3 h, then at 25 °C for 16 h. After evaporation of the solvent in vacuo, the residue was re-dissolved in EtOAc (400 mL), washed with 1 N aqueous $KHSO_4$ (100 mL), 10% aqueous $NaHCO_3$ (100 mL), brine (100 mL) and dried over $MgSO_4$. After evaporation of the solvent in vacuo, the residue was washed with petroleum ether to give a yellowish solid which was refluxed for 2 h in 1 N aqueous NaOH (200 mL) and stirred at 25 °C for 2 h. The solution was cooled to 0 °C. Following addition of 1 N aqueous $KHSO_4$ (250 mL), the mixture was stirred at 0 °C for 30 min, giving rise to the precipitation of acid **13**. After filtration, the residue was washed with small portions of water until the filtrate was neutral (pH=7) and dried in vacuo for 12 h to give acid **13** (14.8 g, 78% over two steps) as a white solid.

R_f=0.28 (CH_2Cl_2/MeOH/HOAc 100/10/1); HPLC R_t

(method 1) = 8.79 min; mp 111–120 °C; HRMS (ES^+): *m/z* calcd for $C_{27}H_{24}N_5O_3$ [M + H^+] 466.1874, found 466.1871; ^1H (300 MHz, d_6-DMSO): δ = 8.15 (s, 1H, CH_{pur}), 7.88 (s, 1H, CH_{pur}), 7.30–7.20 (m, 12H, CH_{Mmt}), 6.85 (d, 2H, ^3J(H,H) = 8.9 Hz, C*H*$COCH_3$), 4.80 (s, 2H, CH_2COO) 3.72 (s, 3H, H_3CO–Ar) ppm; ^{13}C NMR (75 MHz, d_6-DMSO): δ = 169.2 (CO), 157.7 (C), 153.3 (C), 151.0 (CH_{Pur}), 148.9 (C), 145.2 (C), 142.3 (CH_{Pur}), 137.1 (C), 129.8 (CH_{Mmt}), 128.4 (CH_{Mmt}), 127.7 (CH_{Mmt}), 126.5 (CH_{Mmt}), 119.9 (C), 113.0 (CH_{Mmt}), 69.9 ($C(Ar)_3$), 55.0 (CH_3O), 45.0 (CH_2).

4.1.4. Methyl N^2-(4-methoxytrityl)-amino-6-chloro-purin-9-yl acetate (16). To a solution of ester **15**[7] (5 g, 20 mmol) and 4-methoxytrityl chloride (9.58 g, 30 mmol) in dry THF (135 mL) was added DIPEA (3.6 mL, 20 mmol) and the mixture was stirred under a N_2 atmosphere for 12 h. Following evaporation of the solvent, the mixture was taken up in Et_2O (150 mL) and washed with 1 M $KHSO_4$ (3×25 mL); 10% aqueous $NaHCO_3$ (3×25 mL) and brine (1×25 mL). The organic layer was dried over Na_2SO_4 and concentrated in vacuo to give oil which was triturated with petroleum ether 40–60 °C to give ester **16** as a yellowish solid (6.2 g, 60%). R_f = 0.5 (EtOAc); HPLC (method 1) R_t = 10.00 min; mp 55 °C; MS (ES+): *m/z* calcd for $C_{28}H_{25}ClN_5O_3$ [M + H^+] 514.16 found 514.2 [^{35}Cl]; 516.2 [^{37}Cl] ^1H NMR (400 MHz, $CDCl_3$): δ = 7.67 (s, 1H, CH_{pur}), 7.35–7.24 (m, 12H, CH_{Mmt}), 6.81 (d, 2H, ^3J(H,H) = 8.6 Hz, C*H*$COCH_3$), 6.65 (s, 1H, NH-Mmt), 4.37 (s, 2H, CH_2), 3.81 (s, 3H, H_3CO–Ar), 3.67 (s, 3H, $COOCH_3$) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 166.8 (CO), 158.2 (C), 157.6 (C), 152.7 (C), 150.5 (C), 145.0 (C), 141.5 (CH_{pur}), 137.1 (C), 130.2 (CH_{Mmt}); 128.9 (CH_{Mmt}), 127.6 (CH_{Mmt}), 126.5 (CH_{Mmt}), 124.0 (C), 112.9 (CH_{Mmt}), 70.8 ($C(Ar)_3$), 55.3 (H_3CO–Ar), 52.9 ($COOCH_3$), 43.8 (CH_2) ppm.

4.1.5. [N^2-(4-Methoxytrityl)-guanin-9-yl]-acetic acid (17). Ester **16** (6.2 g, 12 mmol) was suspended in 2 N aqueous NaOH (150 mL) and the mixture was stirred for 4 h at reflux. The reaction was cooled to room temperature and acidified with 2 N aqueous HCl (pH = 2). The precipitated was collected by filtration, washed with water and dried, in vacuo overnight giving a solid which was washed with di*iso*propyl ether to give acid **17** (4.2 g, 72%) as a yellowish solid.; R_f = 0.12 (CH_2Cl_2/MeOH 7/3); HPLC (method 1) R_t = 7.50 min; mp >200 °C; HRMS (ES^+) calcd for $C_{27}H_{24}N_5O_4$ [M + H^+] 482.1828; found 482.1831; ^1H NMR (400 MHz, d_6-DMSO): δ = 11.18 (s, 1H, NH_{pur}), 8.06 (s, 1H, NH-Mmt), 8.04 (s, 1H, CH_{pur}), 7.27–7.16 (m, 12H, CH_{Mmt}), 6.81 (d, 2H, ^3J(H,H) = 8.6 Hz, C*H*$COCH_3$), 4.28 (s, 2H, CH_2), 3.72 (s, 3H, H_3CO–Ar) ppm; ^{13}C NMR (100 MHz, d_6-DMSO): δ = 168.5 (CO), 168.0 (CO), 157.7 (C), 155.2 (C), 151.6 (C), 149.1 (C), 148.0 (C) 144.6 (C), 138.0 (CH_{pur}), 136.6 (C); 129.8 (CH_{Mmt}), 128.3 (CH_{Mmt}), 127.4 (CH_{Mmt}), 126.4 (CH_{Mmt}), 112.8 (CH_{Mmt}), 69.9 ($C(Ar)_3$), 55.0 (H_3CO–Ar), 43.8 (CH_2) ppm.

4.1.6. Methyl *N*-{2-[N'^6-(4-methoxytrityl)-adenin-9-yl] acetyl}-*N*-{2-[1-(4,4-dimethyl-2,6-dioxo-cyclohexyliden) ethylamino]ethyl}glycinate (18A(Mmt)). To a solution of acid **13** (5.10 g, 10.9 mmol) and amine **5** (3.38 g, 10.9 mmol) in DMF (25 mL) at 0 °C was added PyBroP (5.07 g, 10.9 mmol) and DIPEA (3.7 mL, 21.8 mmol) at 0 °C, and the reaction was stirred under N_2 at 25 °C for 2 h. The solvent was removed in vacuo, the residue was re-dissolved in CH_2Cl_2 (300 mL), washed with 1 M $KHSO_4$ (100 mL), 10% aqueous $NaHCO_3$ (100 mL) and brine (100 mL). The organic phase was dried over $MgSO_4$ and concentrated in vacuo to give ester **18A(Mmt)** as a brown solid which was used in the next step without purification. An analytical sample was obtained by chromatography.

R_f = 0.62 (CH_2Cl_2/MeOH 5/1); HPLC (method 1) R_t = 9.60 min; mp 125–135 °C; HRMS (ES^+): *m/z* calcd for $C_{42}H_{46}N_7O_6$ [M + H^+] 744.3504, found 744.3510; ^1H (300 MHz, d_6-DMSO) two rotamers: δ = 13.28 and 13.17 (s, 1H, Dde-NH), 8.09 and 8.08 (s, 1H, CH_{pur}), 7.88 (s, 1H, CH_{pur}), 7.31–7.22 (m, 12H, CH_{Mmt}), 6.85 (d, 2H, ^3J(H,H) = 8.5 Hz, CH_{Mmt}), 5.26 and 5.09 (s, 2H, CH_2COO), 4.52 and 4.13 (s, 2H, CH_2CO), 3.79 (m, 2H, CH_2N), 3.74 (s, 3H, CH_3O), 3.72 (s, 3H, CH_3O), 3.57 (m, 2H, CH_2N), 2.57 and 2.46 (s, 3H, CH_3C), 2.30 and 2.27 (s, 4H, CH_2-Dde), 0.94 (s, 6H, CH_3-Dde) ppm; ^{13}C NMR (75 MHz, d_6-DMSO) two rotamers: δ = 196.6 and 196.4 (CO), 173.2 (CO), 169.8 (CO), 169.3 (CO), 167.7 and 167.1 (C), 157.7 (C), 153.4 (C), 151.1 (CH), 149.0 (C), 145.2 (C), 142.3 and 142.1 (CH), 137.1 (C), 129.8 (CH), 128.4 (CH), 127.7 (CH), 126.5 (CH), 119.7 (C), 113.0 (CH), 107.4 and 107.2 (C), 69.9 ($C(Ar)_3$), 55.0 (CH_3), 52.4 (CH_2), 51.8 (CH_3), 49.0 and 48.1 (CH_2), 45.8 (CH_2), 43.7 (CH_2), 41.0 (CH_2), 29.7 (C), 27.8 (CH_3), 17.3 and 17.1 (CH_3) ppm.

4.1.7. Methyl *N*-{2-[N'^4-(4-methoxytrityl)-cytosin-1-yl]-acetyl}-*N*-{2-[1-(4,4-dimethyl-2,6-dioxo-cyclohexyliden) ethylamino]ethyl}-glycinate (18C(Mmt)). This compound was synthesized in analogy to ester **18A(Mmt)**.

R_f = 0.21 (EtOAc); HPLC (method 2) R_t = 3.63 min; mp 135 °C; HRMS (ES+): *m/z* calcd for $C_{41}H_{46}N_5O_7$ [M + H^+] 720.3397, found 720.3402; ^1H NMR (400 MHz, $CDCl_3$): two rotamers δ = 13.50 and 13.37 (br s, 1H, Dde-NH), 7.23–7.05 (m, 12 H, CH_{Mmt}, ^1H CH_{Cyt}), 6.76 (d, ^3J(H,H) = 9.0 Hz, 2H, CH_{Mmt}), 4.99 (d, ^3J(H,H) = 7.8 Hz, 1H, CH_{Cyt}), 4.53 and 4.36 (s, 2H, CH_2COO), 4.27 and 4.02 (s, 2H, CH_2CO), 3.72–3.63 (m, 3H, CH_3O, 4H, CH_2N), 3.52 (CH_3O), 2.51 and 2.47 (s, 3H, CH_3C), 2.25 and 2.24 (s, 4H, CH_2-Dde), 0.95 and 0.93 (s, 6H, CH_3-Dde); ^{13}C NMR (100 MHz, $CDCl_3$) two rotamers: 199.0 and 198.6 (CO), 175.7 (CNH), 170.8 and 170.4 (CO), 168.8 and 168.0 (CO), 163.8 (C), 159.8 (C), 153.0 (CO), 148.2 and 147.2 (CH), 145.1 (C), 136.7 (C), 130.2 (CH), 129.6 (CH), 129.8 (CH), 128.8 (CH), 128.6 (CH), 114.7 and 114.2 (CH), 95.9 (CH), 71.8 ($C(Ar)_3$), 56.3 (H_3C), 53.9 and 53.5 (CH_2), 51.9 and 51.3 (CH_2), 49.9 and 49.5 (CH_2), 47.4 and 47.3 (CH_2), 42.6 and 41.8 (CH_2), 31.0 (C), 29.2 (CH_3), 18.9 and 18.7 (CH_3).

4.1.8. Methyl *N*-[N'^2-(4-methoxytrityl)-guanin-9-yl]-*N*-[2-[1-(4,4-dimethyl-2,6-dioxacyclohexylidene) ethyl-amino]ethyl]glycinate (18G(Mmt)). This compound was synthesized in analogy to ester **18A(Mmt)**.

18 G(Mmt) was obtained as a brown solid (48%) R_f = 0.31 (CH_2Cl_2/MeOH 5/1); HPLC (method 1) R_t = 8.42 min; mp 142–145 °C; HRMS (ES^+) *m/z* calcd for $C_{42}H_{46}N_7O_7$ 760.3459 found 760.3460 [M + H^+] ^1H NMR (400 MHz,

d_6-DMSO) two rotamers $\delta=13.16$ and 13.12 (br s, 1H, Dde-NH), 10.58 and 10.55 (br s, 1H, NH_{pur}), 7.58 and 7.55 (s, 1H, NH-Mmt), 7.44 and 7.42 (s, 1H, CH_{pur}), 7.28–7.12 (m, 12H, CH_{Mmt}), 6.83 (d, ^3J(H,H)=8.5 Hz, 2H, CH_{Mmt}), 4.43 and 4.24 (s, 2H, CH_2COO), 4.10 and 4.05 (s, 2H, CH_2CO), 3.72–3.67 (m, 4H, N–CH_2CH_2-N), 3.66 and 3.61 (s, 3H, CH_3O), 3.43 (s, 3H, CH_3O), 2.49 and 2.47 (s, 4H, CH_2-Dde), 2.30 and 2.26 (s, 3H, CH_3C), 0.95 and 0.94 (s, 6H, CH_3-Dde); ^{13}C NMR (100 MHz, d_6-DMSO): $\delta=196.4$ and 196.2 (CO), 173.0 and 172.7 (CO), 169.4 and 169.2 (CO), 166.9 (CO), 166.3 (C), 157.5 (C), 156.4 (C), 150.8 and 150.7 (C), 149.9 (C), 144.8 (C), 136.7 (CH_{pur}), 129.7 and 129.6 (CH); 128.8 (CH), 127.6 and 127.4 (CH), 126.3 and 126.3 (CH), 116.1 and 116.0 (C), 112.7 (CH), 107.2 (C), 69.7 and 69.6 ($C(Ar)_3$), 54.8 (CH_3), 52.2 (CH_2), 51.7 (CH_3), 48.8 (CH_2), 48.1 (CH_2), 46.5 (CH_2), 40.7 (CH_2), 29.6 (C) 27.7 (CH_3), 17.2 and 17.0 (CH_3) ppm.

4.1.9. *N*-{2-[*N′*^{6}-(4-Methoxytrityl)-adenin-9-yl]-acetyl}-*N*-{2-[1-(4,4-dimethyl-2,6-dioxo-cyclohexyliden)-ethyl-amino]-ethyl}-glycine (19A(Mmt)). Crude ester **18A**(**Mmt**) was suspended in a 1:1 (v/v) mixture of MeOH and 2 M Cs_2CO_3 (60 mL) and stirred for 1.5 h. MeOH was evaporated in vacuo. The remaining solution was diluted with 200 mL water, washed with EtOAc (100 mL), acidified with 1 M aqueous $KHSO_4$, extracted with CH_2Cl_2 (3×200 mL). The organic extracts were washed with brine (100 mL) and the solvent was evaporated in vacuo. The residue was dissolved in a minimum amount of EtOAc. After addition of an excess of hexane, the precipitated solid was collected and dried in vacuo. This precipitate was dissolved in a minimum amount of MeOH. After addition of excess of water, the precipitated solid was collected and dried in vacuo to give acid **19A**(**Mmt**) (3.55 g, 45%) as an off-white solid.

$R_f=0.24$ (CH_2Cl_2/MeOH/HOAc 100/10/1); HPLC (method 1) $R_t=9.01$ min; mp 180 °C (dc); HRMS (ES^+): *m/z* calcd for $C_{41}H_{44}N_7O_6$ [M+H$^+$] 730.3348, found 730.3346 ^{1}H (300 MHz, d_6-DMSO): $\delta=13.26$ and 13.16 (s, 1H, Dde-NH), 8.09 and 8.08 (s, 1H, CH_{pur}), 7.87 (s, 1H, CH_{pur}), 7.31–7.21 (m, 12H, CH_{Mm}), 6.85 (d, 2H, ^3J(H,H)=8.8 Hz,, CH_{Mmt}), 5.24 and 5.07 (s, 2H, CH_2COO), 4.40 and 4.03 (s, 2H, CH_2CO), 3.77 (s, 2H, CH_2N), 3.72 (s, 3H, CH_3O), 3.53 (m, 2H, CH_2N), 2.56 and 2.46 (s, 3H, CH_3CN), 2.29 and 2.26 (s, 4H, CH_2CO), 0.93 (s, 6H, CH_3) ppm; ^{13}C NMR (75 MHz, d_6-DMSO): $\delta=196.4$ (CO), 173.2 (CO), 170.7 (CO), 170.2 (CO), 167.7 and 166.9 (C), 157.7 (C), 153.4 (C), 151.1 (CH), 149.0 (C), 145.2 (C), 142.4 (CH), 137.1 (C), 129.8 (CH), 128.5 (CH), 127.7 (CH), 126.5 (CH), 119.7 (C), 113.0 (CH), 112.2 (C), 69.9 (C), 55.0 (CH_3), 52.4 (CH_2), 49.2 (CH_2), 48.0 and 46.6 (CH_2), 43.7 (CH_2), 40.9 (CH_2), 29.7 (C), 27.8 (CH_3), 17.3 and 17.1 (CH_3).

4.1.10. *N*-{2-[*N′*^{4}-(4-Methoxytrityl)-cytosin-1-yl]-acetyl}-*N*-{2-[1-(4,4-Dimethyl-2,6-dioxo-cyclohexyliden)-ethyl-amino]-ethyl}-glycine (19C(Mmt)). This compound was synthesized in analogy to acid **19A**(**Mmt**).

$R_f=0.37$ (BuOH/HOAc/MeOH 3/1/1); HPLC (method 2) $R_t=3.52$ min; mp 174 °C; HRMS (ES+): *m/z* calcd for $C_{40}H_{43}N_5O_7$ [M+Na$^+$] 728.3055, found 728.3034^{1}H NMR (400 MHz, CD_3OD)= two rotamers $\delta=7.42$–7.25 (m, 10H, CH_{Mmt}, 1H, CH_{Cyt}), 7.20 (d, 2H ^3J(H,H)=8.5 Hz, CH_{Mmt}), 6.90 (d, ^3J(H,H)=9.0 Hz, 2H, CH_{Mmt}), 5.30 and 5.26 (d, ^3J(H,H)=7.5 Hz, 1H, CH_{Cyt}), 4.71 and 4.57 (s, 2H, CH_2COO), 4.26 and 4.12 (s, 2H, CH_2CO), 3.90–3.66 (m, 3H, CH_3O, 4H, CH_2N), 2.56 and 2.53 (s, 3H, CH_3C), 2.33 and 2.29 (s, 4H, CH_2-Dde), 0.98 and 0.96 (s, 6H, CH_3-Dde) ppm; ^{13}C NMR (100 MHz, CD_3OD) two rotamers: 198.9 and 198.6 (CO), 174.8 (CNH), 171.4 and 171.1 (CO), 168.8 and 1678.0 (CO), 163.8 (C), 159.5 (C), 153.5 (CO), 148.8 (CH), 143.6 (C), 135.1 (C) 129.9 (CH), 128.5 (CH), 128.1 (CH), 127. (CH), 113.2 (CH), 95.1 (CH), 71.6 ($C(Ar)_3$), 56.3 (H_3C), 53.9 (CH_2), 51.2 and 50.9 (CH_2), 50.0 (CH_2), 49.2 and 49.0 (CH_2), 42.9 and 42.7 (CH_2), 29.9 (C), 27.4 (CH_3), 17.4 and 17.2 (CH_3) ppm.

4.1.11. *N*-[*N′*^{2}-(4-Methoxytrityl)guanin-9-yl]-*N*-[2-[1-(4,4-dimethyl-2,6-dioxacyclohexylidene)ethylamino] ethyl]glycine (19G(Mmt)). To a solution of ester **18G**(**Mmt**) (2.5 g, 3.3 mmol) in methanol (16.5 mL) was added dropwise a 2 M Cs_2CO_3 aqueous solution (16.5 mL). The solution was stirred for 1.30 h. MeOH was evaporated in vacuo and the remaining solution was diluted with water (50 mL) washed with CH_2Cl_2 (3×15 mL). The basic solution was brought to pH 2 using 1 M $KHSO_4$. The precipitate was collected by filtration, washed with water and re-dissolved in a minimum of MeOH. Following addition of an excess of water, the precipitated solid was collected and dried in vacuo to give acid **19G**(**Mmt**) (1.4 g, 57%) as a brown solid $R_f=0.24$ (BuOH/HOAc/H_2O 3/1/1); HPLC (method 1) R*t*=7.072 min; mp 175 °C(dc); HRMS (ES^+) calcd for $C_{41}H_{44}N_5O_4$ [M+H$^+$]746.3297, found 746.3280; ^{1}H NMR (400 MHz, d_6-DMSO): two rotamers $\delta=13.14$ and 13.13 (br s, 1H, Dde-NH), 10.50 (br s, 1H, NH_{pur}), 7.5 and 7.55 (s, 1H, NH-Mmt), 7.46 and 7.44 (s, 1H, CH_{pur}), 7.25–7.13 (m, 12H, CH_{Mmt}), 6.83 (d, ^3J(H,H)= 8.7 Hz, 2H, CH_{Mmt}), 4.45 and 4.22 (s, 1H, CH_2COO), 3.98 and 3.93 (s, 1H, CH_2CO), 3.73–3.70 (m, 4H, NCH_2CH_2N), 3.41 (s, 3H, CH_3O), 2.50 and 2.47 (s, 4H, CH_2-Dde), 2.31 and 2.27 (s, 3H, CH_3C), 0.95 and 0.94 (s, 6H, CH_3-Dde) ^{13}C NMR (100 MHz, d_6-DMSO) two rotamers: $\delta=196.4$ and 196.2 (CO), 172.9 and 172.7 (CO), 170.4 and 170.2 (CO), 167.0 (CO), 166.1 (C), 157.5 (C), 156.4 and 156.4 (C), 150.8 and 150.6 (C), 149.7 (C), 144.8 (C), 138.1 and 137.8 (CH_{pur}), 129.7 and 129.6 (CH); 128.3 and 128.3 (CH), 127.4 (CH), 126.3 (CH), 116.1 and 116.0 (C), 112.7 (CH), 107.2 and 107.1 (C), 69.7 and 69.5 ($C(Ar)_3$), 54.8 (CH_3), 52.3 (CH_2), 49.3 and 49.3 (CH_2), 48.1 (CH_2), 46.6 and 46.5 (CH_2), 42.7 and 42.5 (CH_2), 40.7 (CH_2), 29.6 (C), 27.7 and 27.7 (CH_3), 17.2 and 17.0 (CH_3) ppm.

4.1.12. *N*-[2-(Thymin-1-yl)-acetyl]-*N*-{2-[1-(4,4-dimethyl-2,6-dioxo-cyclohexyliden)-ethylamino]-ethyl}-glycine (19T). To a solution of amine **5** (4.5 g, 15.1 mmol) in EtOAc (17 mL) was added propylphosphonic acid cyclic (50% solution in DMF) (17 mL, 15.7 mmol), acid **7** (2.9 g, 15.7 mmol), and DIPEA (5.57 mL, 32 mmol and the reaction was stirred for 16 h. Then a mixture of ice-cold water (650 mL) and saturated $NaHCO_3$ solution (350 mL) was added and stirred. The mixture was extracted with EtOAc (100 mL ×9). The organic layers were dried over $MgSO_4$ and concentrated to give a crude product which was reprecipitated using EtOAc/diisopropyl ether mixture. The

solid was collected by filtration to give ester **18T** (4.5 g, 65%) as a white solid.

Crude **18T** (4.5 g, 9 mmol) was suspended in a 1:1 (v/v) mixture of MeOH and 2 M Cs_2CO_3 (90 mL) and stirred for 1.5 h. The MeOH was evaporated in vacuo, the aqueous phase was acidified with 4 N HCl (pH=1), the solvents were removed in vacuo. The residue was treated with hot 2-propanol and the hot suspension was filtrated. The filtrate was collected and 2-propanol was removed in vacuo. The residue was sonicated with water (25 mL) and filtrated to give acid **19T** (3.1 g, 77%) as a white solid.

R_f=0.13 (CH_2Cl_2/MeOH/HOAc 100/10/1); HPLC (method 1) Rt=7.59 min; mp 243–247 °C; HRMS (ES^+): *m/z* calcd for $C_{21}H_{28}NaN_4O_7$ [M+Na^+] 471.1850, found 471.1846; ^1H (300 MHz, d_6-DMSO) two rotamers: δ=13.15/13.10 (m, 1H, Dde-NH), 11.30 and 11.27 (s, 1H, COOH), 7.32 and 7.30 (d, 1H, ^4J(H,H)=1.5 Hz, CH), 4.64 and 4.48 (s, 2H, CH_2COO), 4.26 and 3.99 (s, 2H, CH_2CO), 3.60–3.70 (m, 2H, CH_2N), 3.43–3.57 (m, 2H, CH_2N), 2.50 and 2.45 (s, 3H, CH_3C), 2.26 and (s, 4H, CH_2-Dde), 1.72 (s, 6H, CH_3-Dde), 0.91 and 0.90 (s, 6H, CH_3-Dde) ppm; ^{13}C NMR (75 MHz, CD_3COOD): δ=199.7 and 199.5 (CO), 175.8 (CO), 172.7 and 172.5 (CO), 169.2 and 168.5 (CO), 166.2 (C), 151.8 (CO), 142.8 (CH), 110.5 (C), 107.9 (C), 51.6 (CH_2), 51.4 (CH_2), 49.7 (CH_2), 48.4 and 47.7 (CH_2), 41.5 and 41.0 (CH_2), 29.7 (C), 27.1 (CH_3), 17.8 and 17.6 (CH_3), 11.1 (CH_3).

4.1.13. Fmoc-Lys(Dde)-Rink amide PEGA resin (22). Fmoc-Lys(Dde)-OH **21**[15] (851 mg, 1.6 mmol) and HOBt (211 mg, 1.6 mmol) were dissolved in DMF (16 mL) and shaken for 10 min. DIC (0.296 mL, 1.92 mmol) was added to the mixture and stirred for a further 10 min. The resulting solution was added to the PEGA Rink amine resin (10 g of wet resin, loading 0.04 mmol/g) which was pre-swollen in CH_2Cl_2 (3×25 mL). The mixture was shaken overnight and the progress of the reaction was monitored by a qualitative ninhydrin test. The resin was filtered and washed thoroughly with DMF (3×20 mL), CH_2Cl_2 (3×20 mL) and MeOH (3×20 mL). An analytical sample was taken and cleaved using TFA/TIS (95:5) to give Fmoc-Lys(Dde)-NH_2. HPLC (method 1) R_t=9.62 min; MS (ES^+): *m/z* calcd for $C_{31}H_{38}N_3O_5$ [M+H^+] 532.3, found 532.3.

4.1.14. Fmoc-Lys-Rink amide PEGA resin (23). Resin **22** was pre-swollen in DMF (3×25 mL) before adding a 20% solution of imidazole/hydroxylamine hydrochloride (1/0.75 equiv) in NMP/DMF (5/1, 25 mL).[11] The resulting mixture was shaken for 1 h and the resin was filtered and washed thoroughly with DMF (3×20 mL), CH_2Cl_2 (3×20 mL) and MeOH (3×20 mL). An analytical sample was taken and coupled to Fmoc-Gly-OH (see general procedure for amino acids coupling) before cleavage was carried out using TFA/TIS (95:5) to give Fmoc-Lys(Fmoc-Gly)-NH_2. HPLC (method 1) R_t=10.49 min; MS (ES^+): *m/z* calcd for $C_{38}H_{38}NaN_4O_6$ [M+Na^+] 669.2, found 669.22.

4.2. General procedure for PNA monomer couplings

Dde PNA monomer **19** (5.5 equiv) and PyBOP (5 equiv) were dissolved in DMF followed by the addition of NEM (11 equiv). The resulting solution was mixed for 10 sec. before adding to the resin (1 equiv) pre-swollen in DMF and the mixture was then shaken for 3 h. Resins were washed with DMF (3×3 mL), CH_2Cl_2 (3×3 mL) and MeOH (3×3 mL),

4.3. General procedure for amino acids couplings

Fmoc amino acids (5.5 equiv), PyBOP (5 equiv) and HOBt (5 equiv) were dissolved in DMF followed by addition of DIPEA (11 equiv). The resulting solution was mixed for 10 s. before adding to resins (1 equiv) pre-swollen in DMF and the mixture was then shaken for 3 h. Resins were washed with DMF (3×3 mL), CH_2Cl_2 (3×3 mL) and MeOH (3×3 mL),

4.3.1. Conjugate 24. HPLC (method 3) R_t=13.75 min; MALDI-TOF: *m/z* calcd for $C_{21}H_{28}NaN_4O_7$ (average mass) [M+H^+] 4327.21 found 4237.45.

4.3.2. Conjugate 26. HPLC (method 3) R_t=12.26 min; MALDI-TOF: *m/z* calcd for $C_{179}H_{227}N_{72}O_{48}$ (average mass) [M+H^+] 4155.1 found 4157.4.

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