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Synthesis of hydrazino-peptide nucleic acid monomers and dimers as new PNA backbone building blocks

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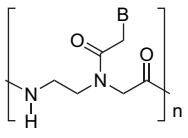
Abstract—We describe the synthesis of new hydrazinoPNA (*hyd*PNA) monomers and new *hyd*PNA-containing dimers. For the *hyd*PNA monomers, the primary terminal amino group of the aminoethylglycine unit of classical *aeg*PNA is replaced by a hydrazine moiety. An appropriate choice of two orthogonal protecting groups on the two hydrazine nitrogen atoms makes it possible to drive their coupling with other monomers selectively on one or the other nitrogen atom, thus obtaining two different types of PNA dimers. These dimers represent new building blocks that can be used to generate novel PNA oligomers.

1. Introduction

Peptide nucleic acid (PNA) is an artificial DNA mimic introduced by Nielsen in 1991,[1,2] which has a pseudopeptide backbone replacing the sugar-phosphate chain. The backbone is made of *N*-(2-aminoethyl)glycine units linked in a polyamide structure, and the purine (A, G) and pyrimidine nucleobases (C, T) are linked to the α-nitrogen atom of the amino acid unit through methylene carbonyl residues. In this way, the repeating unit consists of six atoms, exactly as in DNA and RNA.



PNA
B = nucleobase

Although its backbone structure is completely different from that of natural nucleic acids, *N*-(2-aminoethyl)glycine PNA (*aeg*PNA) has high binding affinity and specificity[3–6] for the complementary single strands of DNA (ssDNA) and RNA, which is at least partially attributable to the absence of electrostatic repulsion between the neutral molecule of PNA and the polyanionic target DNA and RNA. For these reasons, PNA is a very interesting and potentially useful tool in molecular biology, and may have both diagnostic and therapeutic applications.[7–9]

Within the framework of a research project aimed at designing and synthesising new PNA monomers, we have recently prepared some organometallic conjugates (Fig. 1) in which a Fischer-type chromium carbene,[10,11] a tricarbonylchromium-arene moiety[12] and one or more ferrocenyl unit[13,14] have been introduced as potential spectroscopic and electrochemical probes. All of these new bio-organometallic conjugates have interesting spectroscopic and electrochemical properties,[15] and are therefore potentially useful for diagnostic purposes.[16]

Despite these interesting and useful properties, a number of drawbacks justify the search for new PNA mimics that overcome the existing limited water solubility,[17] tendency to self-aggregation and poor cell permeability.[18]

Another quite important aspect is the presence of molecular constraints within the backbone that can influence the efficiency and specificity of PNA–DNA binding. A number of studies have been carried out in an attempt to increase the binding affinity of PNA to complementary DNA and RNA by modifying the rigidity of the PNA backbone, for example, Nielsen et al.[19] have reported that the flexibility of a PNA backbone bearing a tertiary amine instead of the tertiary amide reduces DNA binding affinity, whereas Appella

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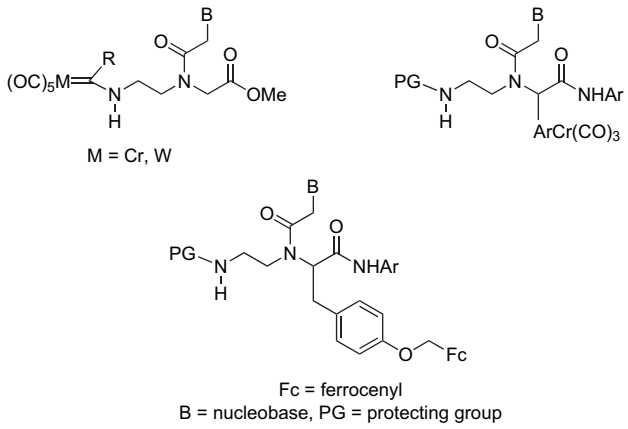


Figure 1. Organometallic conjugates of PNA monomers.

et al.[20] have recently found that a PNA decamer in which the secondary amide bond is replaced by a more flexible secondary amine has the same binding affinity for the complementary DNA strand as the unmodified oligomer.

Marchelli et al. showed that a PNA oligomer containing three modified monomers with chiral lysines (a chiral box) is excellent at discriminating mismatched and matched targets; moreover, the chiral box allowed the formation of the anti-parallel PNA–DNA duplex, whereas the parallel PNA–DNA duplex failed to form.[21,22] The molecular basis of this selectivity was described in detail by solving the crystal structure of the PNA–DNA duplex.[23]

These results indicate that a chiral constraint in the middle of a PNA sequence greatly affects direction selectivity in DNA complexation. The effect of the substitution of the α- or γ-carbon of the PNA backbone,[24] and its stereochemistry,[25] has more recently been studied in detail. The relevance of these studies is well exemplified by the fact that some of these modified PNAs have been used in cell systems, such as tumoral or stem cells,[26,27] or in advanced molecular diagnostics.[28,29]

However, as no conclusive data are yet available concerning the importance of other molecular constraints in the PNA backbone, research in this field still seems to be useful.

In an attempt to contribute towards solving some of the above problems, we have designed new PNA monomers; the hydrazinoPNA[30] of general formula **1** (*hyd*PNA, Fig. 2), in which the terminal amino group of *aeg*PNA is replaced by a hydrazine moiety that can be protected by two orthogonal protecting groups at the nitrogen atoms.

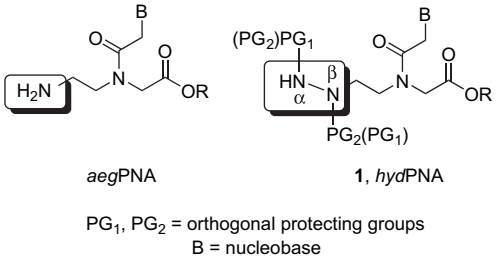


Figure 2. Aminoethylglycine- and hydrazinoPNA (*aeg*- and *hyd*PNA) monomers.

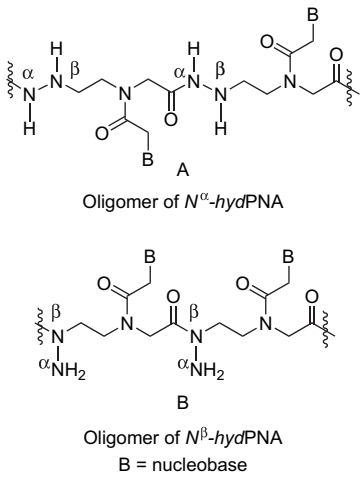


Figure 3.

In principle, *hyd*PNA monomers could be oligomerised as such in order to obtain type **A** and **B** homo-oligomers of *hyd*PNA (Fig. 3), or could be inserted as co-monomers in *aeg*PNA oligomers. In both cases, two different oligomerisation sites should be available: the terminal (N^{α}) and the internal (N^{β}) nitrogen atoms of the hydrazine moiety. The appropriate choice of the two protecting groups (PG_1 and PG_2 in Fig. 2) would assure the possibility of driving the growth of the PNA oligomer chain on the terminal (N^{α}) or internal (N^{β}) nitrogen atom. In the first case, type **A** homo-oligomers would be obtained with a seven-atom repetitive unit; in the second case, type **B** oligomers would be formed with a six-atom repetitive unit and pendant amino groups (Fig. 3).

The presence of the additional nitrogen atom should give *hyd*PNA oligomers particular chemico-physical characteristics, such as a reduced loss of entropy in the duplex formation due to the highly expected rigidity of the PNA oligomers of N^{α}-*hyd*PNA and, perhaps, increased water solubility for both oligomers due to the presence of the hydrophilic amino groups. Moreover, the presence of the amino groups in both **A** and **B** should make it possible to obtain poly-cationic PNA oligomers whose binding affinity for complementary ssDNA should be enhanced.

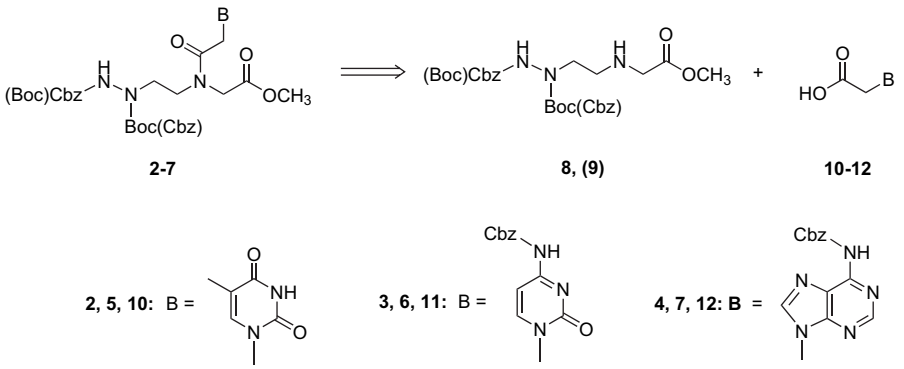
In order to achieve and verify these objectives, we synthesised new *hyd*PNA monomers and looked for the best conditions in which to couple *hyd*PNA and *aeg*PNA, which required the formation of a hydrazide bond in view of the aim to use the resulting dimers as building blocks to make PNA oligomers.

We here describe the optimised synthetic methods for preparing new *hyd*PNA monomers and new *hyd*PNA-containing dimers.

2. Results and discussion

As stated above, the synthesis of oligomers **A** and **B** requires appropriately designed *hyd*PNA monomers.

In particular, obtaining type **A** oligomers requires the use of N^{β}-protected *hyd*PNA monomers in which the unprotected



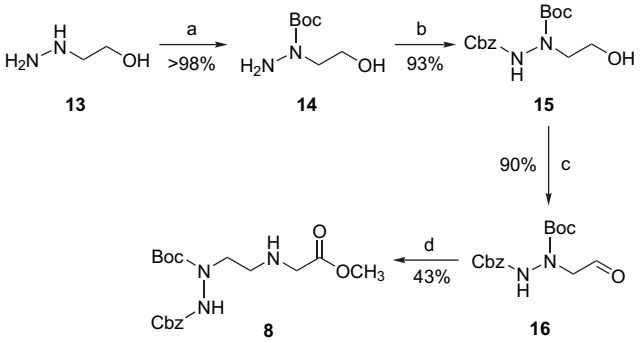
Scheme 1. Retrosynthetic scheme for *hyd*PNA monomers.

terminal amino group (N^{α}) is available for coupling with a carboxylic acid function of a second monomer, whereas obtaining type **B** oligomers requires the preparation of monomers in which the internal nitrogen atom (N^{β}) is free for coupling. This strategy based on the use of orthogonal protecting groups led us to synthesise the new compounds N^{α}-Cbz,N^{β}-Boc **2–4**, and N^{α}-Boc,N^{β}-Cbz **5–7**, substituted with the three nucleobases thymine, cytosine and adenine (Scheme 1).

Using the Boc strategy for oligomerisation (i.e., deprotecting the Boc amino group and growing the oligomer chain upon it), type **B** oligomers (Fig. 3) should be obtainable from monomers **2–4** and type **A** oligomers from monomers **5–7**.

Retrosynthetic analysis of compounds **2–7** led us to synthesise the two backbones **8** and **9** from which to obtain the target new *hyd*PNA monomers **2–7** through the coupling of nucleobases **10–12** (Scheme 1).

The first backbone **8** (with the Cbz group on the terminal nitrogen atom and the Boc group on the internal nitrogen atom) was prepared following the synthetic sequence shown in Scheme 2.



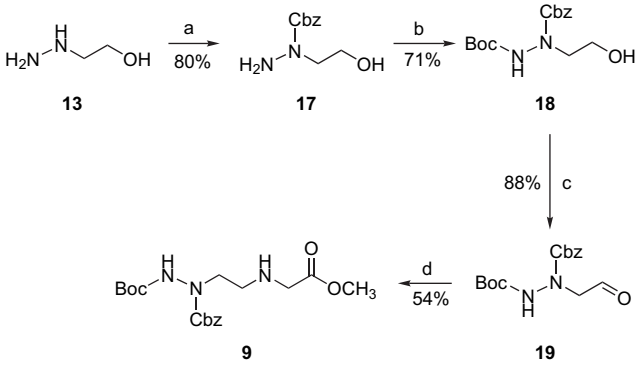
Scheme 2. Reagents: (a) $(Boc)_2O$, EtOH; (b) Cbz-Cl, aq NaOH, CH_2Cl_2; (c) (i) Dess–Martin periodinane, CH_2Cl_2; (ii) $Na_2S_2O_3$, $NaHCO_3$, H_2O, MTBE; (d) $H_2NCH_2CO_2CH_3 \cdot HCl$, Et_3N, $ZnCl_2$, $NaBH_3CN$, MeOH.

The commercially available *N*-2-hydroxyethylhydrazine **13** was chosen as the starting compound. The internal nitrogen atom was protected to give the Boc derivative **14**,[31] and protecting the external amino group with Cbz-Cl gave the orthogonally diprotected compound **15**. The primary alcoholic function of **15** was then oxidised in high yield to the formyl group using Dess–Martin periodinane. The subsequent chain elongation to give the hydrazinoPNA backbone **8** was achieved in high yield by means of the reductive amination of aldehyde **16** with the glycine methyl ester using $NaBH_3CN$ and $ZnCl_2$.[32]

Compounds **15**, **16** and **8** are new, and are completely characterised by means of spectroscopic data.

All of the compounds shown in Scheme 2 were obtained in high yield; no column chromatography purification was necessary for compounds **14** and **15** and so the whole sequence was completed within one week.

Similarly, the *hyd*PNA backbone **9** was synthesised following the strategy shown in Scheme 3.



Scheme 3. Reagents: (a) Cbz-Cl, Et_3N, CH_2Cl_2; (b) $(Boc)_2O$, Et_3N, THF; (c) (i) Dess–Martin periodinane, CH_2Cl_2; (ii) $Na_2S_2O_3$, $NaHCO_3$, H_2O, MTBE; (d) $H_2NCH_2CO_2CH_3 \cdot HCl$, MeOH, $NaBH_3CN$, CH_3COOH.

This time, the first step was to protect the internal nitrogen atom of compound **13** with the Cbz group by reacting **13** with Cbz-Cl, followed by protecting the terminal amino group (N^{α}) with Boc. Oxidation of the obtained alcohol **18** with Dess–Martin periodinane gave the expected aldehyde **19**, which was converted into the backbone **9** by means of reductive amination.

Compounds **18** and **9** are new and are completely characterised by means of spectroscopic data, whereas **17**[33] and **19**[34] have been previously described. Unlike compounds **14** and **15**, the analogous compounds **17** and **18** required column chromatography purification.

At this point, all that was necessary to obtain the target *hyd*PNA monomers **2–7** was to introduce the nucleobases onto the glycine nitrogen atom of **8** and **9**, which was achieved in good yield using standard conditions for coupling the nucleobases with *aeg*PNA.[35] In particular, **8** and **9** were coupled with the 1-carboxymethyl derivatives of nucleobases **10–12** in the presence of 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (DhbtOH), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC·HCl) and diisopropylethylamine (DIEA) (Scheme 4).

The new PNA monomers **2–7**, the first target of our project, constitute the building blocks necessary for the construction of *hyd*PNA oligomers. They can be easily and efficiently obtained using the synthetic methodologies shown in Schemes 2–4.

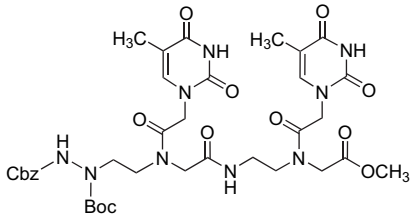
The next step was to set up and optimise the coupling conditions between the *hyd*PNA and *aeg*PNA monomers to give the new dimers **20–22**, which have either a classical amide (compound **20**) or hydrazide-type bond (compounds **21** and **22**) (Fig. 4). This work was much less simple than expected.

In order to synthesise these dimers, it was necessary to find the appropriate conditions for methyl ester hydrolysis and Boc deprotection in the *hyd*PNA monomers.

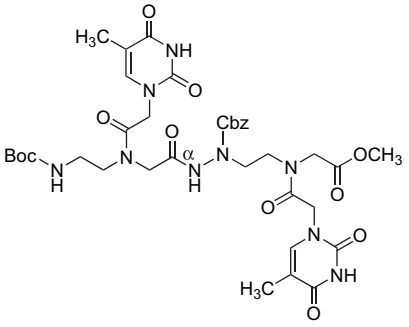
The ester group in compounds **2** and **5** was hydrolysed very efficiently and easily by means of a reaction with aq potassium hydroxide to give the corresponding acids **23** and **24** in high yields (Scheme 5).

The two new *hyd*PNA acids **23** and **24** are stable, and are completely characterised by means of spectroscopic data.

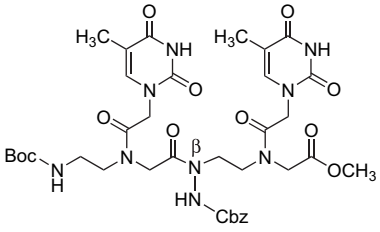
Similarly, in a preliminary study aimed at finding the appropriate conditions for selectively deprotecting the N^{α}- or N^{β}-Boc groups in *hyd*PNA, we found that this could be easily and efficiently achieved using trifluoroacetic acid in dichloromethane. In this way, compounds **2**, **3**, **5** and **6** gave monomers **25–28**, with a free amino group suitable for the following coupling steps (Scheme 6).



20, *hyd*PNA-*aeg*PNA



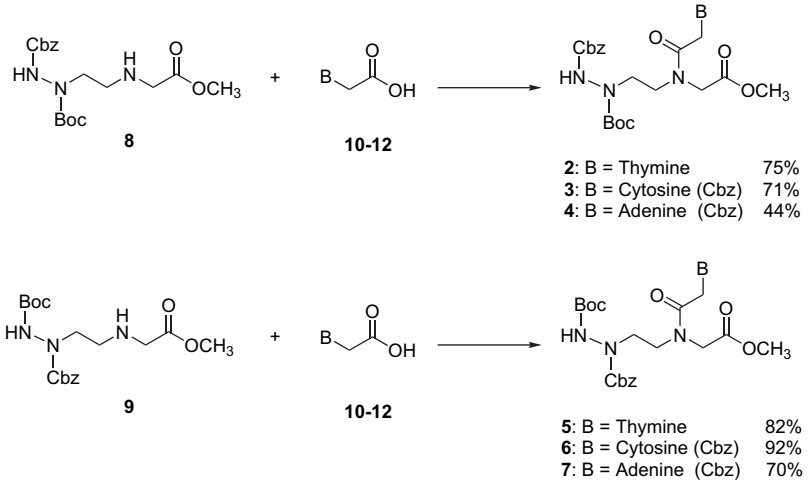
21, *aeg*PNA-N^{α}*hyd*PNA



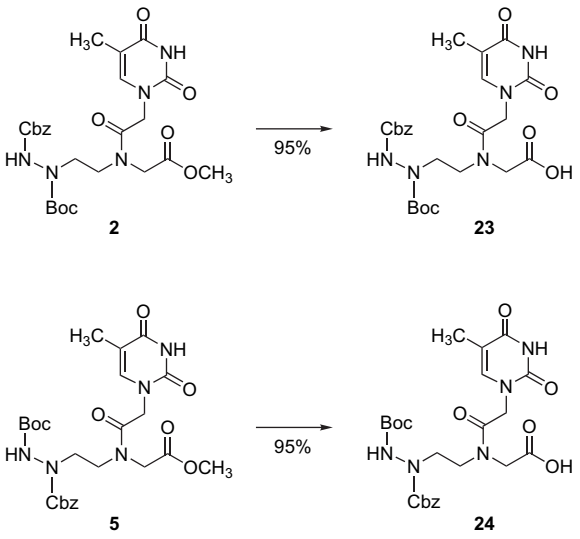
22, *aeg*PNA-N^{β}*hyd*PNA

Figure 4. New PNA dimers.

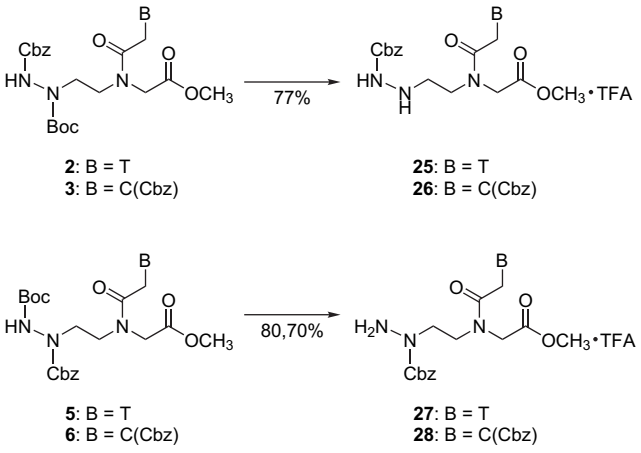
As we were able to deprotect the ester function and N^{α}-Boc or N^{β}-Boc selectively, we set up the conditions to synthesise the target dimers **20–22** between *aeg*PNA and *hyd*PNA. The monomer **23** was reacted with the *aeg*PNA monomer **29** as shown in Scheme 7, and the new dimer **20** was isolated after purification on column chromatography in 92% yield. The



Scheme 4. Reagents and conditions: DhbtOH, DIEA, EDC·HCl, DMF, 16–48 h, rt.



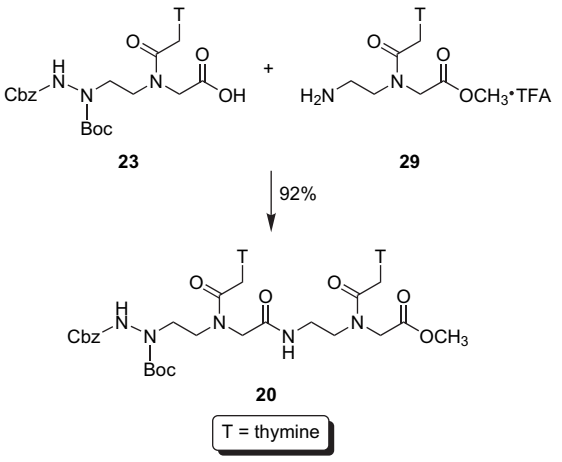
Scheme 5. Reagents: (i) 2 M aq KOH; (ii) 1 M aq HCl.



Scheme 6. Reagents: TFA, CH_2Cl_2.

use of HBTU as the condensing agent instead of DCC and pentafluorophenol (PFP) led to a lower yield (76%).

The new dimer **20** could be deprotected at the ester function by means of LiOH in methanol to give acid **30** in 50% yield, and at the N^{β}-Boc site by means of 20% TFA in CH_2Cl_2 to give **31** in 98% yield (Scheme 8).



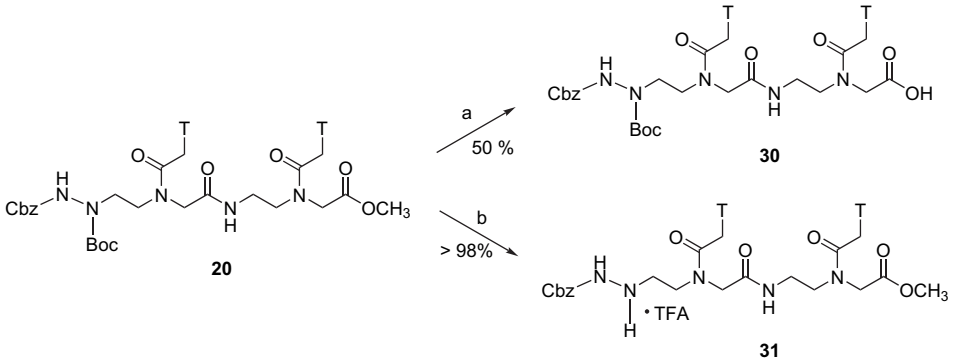
Scheme 7. Reagents: DCC, PFP, DIEA, DMF.

After verifying the good reactivity of the free carboxylic acid group in *hyd*PNA monomer **23** (Scheme 7), we checked the reactivity of the hydrazine moiety at the positions of N^{α}-Boc (compound **5**) and N^{β}-Boc (compound **2**), which were deprotected as shown in Scheme 6 and reacted with Boc-*aeg*PNA-COOH **32** (Scheme 9) to afford dimers **21** and **22** in 65 and 56% yield, respectively.

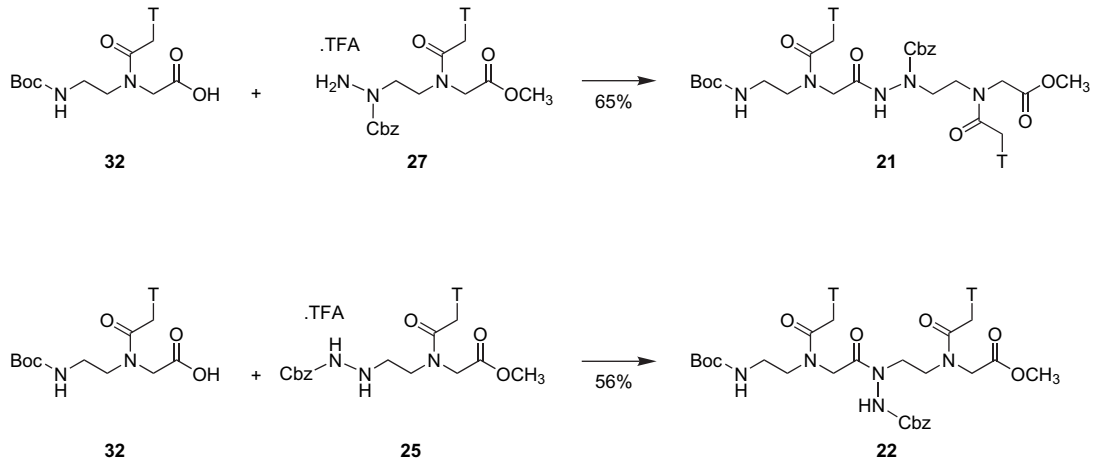
The reaction conditions shown in Scheme 9 are the result of a study carried out in order to optimise the formation of the hydrazide bond. The unprotected amino groups in both *hyd*PNA **25** and **27** (N^{β} and N^{α}, respectively) showed unexpectedly less reactivity than the corresponding amino group of *aeg*PNA and so their reaction with the carboxylic function of *aeg*PNA **32** under the conditions shown in Scheme 7 only gave 5% of the corresponding dimers after 30 h. On the contrary, by coupling **25** and **27** with **32** in the presence of EDC·HCl, DhbtOH and DIPEA (Scheme 9), we could isolate the target dimers **22** and **21** in satisfactory yields, although this required long reaction times (24–48 h). The new dimers, **21** and **22**, are stable crystalline compounds, and have been completely characterised.

3. Conclusions

We here describe the synthetic methodologies for obtaining new PNA monomers with a modified backbone (*hyd*PNA



Scheme 8. Reagents: (a) (i) LiOH, MeOH; (ii) 0.5 M aq $KHSO_4$; (b) TFA, CH_2Cl_2.



Scheme 9. Reagents: EDC·HCl, DhbtOH, DIPEA, dry DMF.

2–**7**) in which the primary terminal amino group of the aminoethylglycine unit of classical *aeg*PNA is replaced by a hydrazine moiety. We have shown that an appropriate choice of the two orthogonal protecting groups on the hydrazine nitrogen atoms makes it possible to drive their coupling with other monomers on one or the other nitrogen atom, thus obtaining two different types of new PNA dimer (**21** and **22**).

All of the monomers and dimers described above are new building blocks that can be used to generate the novel PNA oligomers, whose binding affinity for complementary DNA strands is currently being investigated.

4. Experimental

4.1. General

All reagents were obtained from commercial suppliers and used without further purification. Dry DMF and dry methanol over molecular sieves were obtained from Fluka. THF was dried over sodium/benzophenone. Dess–Martin periodinane was prepared according to the literature.[36] Thymine-1-acetic acid was obtained from Aldrich. Compounds **11** and **12** were prepared according to the literature.[37,38]

Unless otherwise specified, all of the reactions were performed in an inert atmosphere under dry conditions.

The IR spectra were recorded on a Perkin–Elmer FTIR 1725X1. ^{1}H and ^{13}C NMR were recorded on Bruker AC200, AC300 and AMX300 machines, and the chemical shifts (δ) are reported in parts per million relative to solvent peak. Many of the compounds described below, in particular **20**–**22**, **30** and **31**, showed many rotamers and consequently had complex ^{1}H NMR patterns. Some of the signals in the ^{1}H NMR are therefore described as major (ma.) and minor (mi.) components, wherever possible; however, in many cases, some minor signals were obscured by the major signal of another proton. In such cases, only the major signal is reported or a range containing two or more CH_2. In some cases, the ^{13}C spectrum shows many peaks that cannot be distinguished in a small range: ov. means 'overlapped'.

Melting points were obtained by Büchi Melting Point B-540 and (dec) indicates decomposition. Mass spectra were recorded using a Thermo Finnigan LCQ Advantage; high-resolution mass spectra were recorded using a Bruker Daltonics ICR-FTMS APEX II.

4.1.1. Methyl-*N*-[2-(*N*$^{\alpha}$-benzyloxycarbonyl-*N*$^{\beta}$-*tert*-butoxycarbonyl-hydrazino)ethyl]-*N*-[(thymin-1-yl)acetyl]-glycinate (2). To a solution of **8** (394.5 mg, 1.0 mmol) in dry DMF (9 mL) under vigorous stirring, **10** (209 mg, 1.1 mmol), DhbtOH (186 mg, 1.1 mmol) and DIEA (200 μL, 1.2 mmol) were added. The mixture was cooled to 0 °C in an ice bath and EDC·HCl (228 mg, 1.2 mmol) was added portionwise in 30 min. The mixture was allowed to warm to rt. The reaction was stirred for 22 h while monitoring by TLC (eluent: ethyl acetate/petroleum ether 8:2, R_f=0.24). The solvent was removed in vacuo and the residue was partitioned between water (15 mL) and ethyl acetate (35 mL). The organic layer was washed with 0.1 M aq $KHSO_4$ (2×10 mL), water (2×10 mL), satd aq $NaHCO_3$ (4×20 mL) and brine (1×10 mL). The aqueous phases were combined and extracted with ethyl acetate (3×50 mL).

The organic phases were dried over Na_2SO_4, filtered and the filtrate was concentrated to dryness to afford 475 mg of crude product as a dark orange oil. Column chromatography on silica gel (gradient from ethyl acetate/petroleum ether 9:1 to ethyl acetate/methanol 7:3) afforded **2** (427 mg, 75%) as a white solid: mp=89–94 °C (dec); ^{1}H NMR (300 MHz, $CDCl_3$) (two rotamers): δ 9.26 (br s, 1H), 7.83 (s, 1H), 7.39–7.35 (m, 5H), 6.99 (ma.) and 6.95 (mi.) (s, 1H), 5.14 (ma.) and 5.11 (mi.) (s, 2H), 4.52 (ma.) and 4.38 (mi.) (s, 2H), 4.18 (mi.) and 4.07 (ma.) (s, 2H), 3.68 (s, 3H), 3.76 (m, 2H), 3.61 (m, 2H), 1.85 (s, 3H), 1.34 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 169.5, 169.3, 167.2, 164.2, 155.1, 151.1, 141.3, 135.7, 128.4, 110.5, 81.5, 67.5, 52.3, 48.2, 47.6, 47.5, 46.0, 27.9, 12.2; mass spectrum ESI *m*/*z* 570.2143 ($C_{25}H_{33}N_5O_9$+Na requires 570.2176); IR (film, CCl_4, cm^{-1}): 3403, 1754, 1695, 1369, 1110, 1068, 629.

4.1.2. Methyl-*N*-[2-(*N*$^{\alpha}$-benzyloxycarbonyl-*N*$^{\beta}$-*tert*-butoxycarbonyl-hydrazino)ethyl]-*N*-{[4-*N*-(benzyloxycarbonyl)cytosin-1-yl]acetyl}glycinate (3). To a solution of **11**

(135 mg, 0.46 mmol) and **8** (158 mg, 0.41 mmol) in dry DMF (2 mL), DhbtOH (75 mg, 0.46 mmol) and DIEA (80 μL, 0.46 mmol) were added under stirring. The mixture was cooled to 0 °C in an ice bath and then EDC·HCl (88 mg, 0.46 mmol) was added portionwise. The mixture was allowed to warm to rt. The pH was adjusted to 9 with DIEA. The reaction was stirred overnight. The solvent was removed in vacuo and the residue, a yellow oil, was partitioned between water (7.5 mL) and ethyl acetate (17 mL). The organic layer was washed with 0.1 M aq $KHSO_4$ (2×5 mL), water (2×6 mL), satd aq $NaHCO_3$ (4×11 mL) and brine (1×5 mL). All the aqueous phases were combined and extracted with ethyl acetate (2×25 mL). These organic phases were combined with the first and dried over Na_2SO_4. After filtration, the filtrate was concentrated in vacuo to afford 352 mg of crude product as a pale yellow solid. Column chromatography on silica gel (ethyl acetate/petroleum ether 9:1) afforded **3** (196.3 mg, R_f=0.14, 71%) as a white solid: mp=128.6–130.9 °C (dec); ^{1}H NMR (200 MHz, $CDCl_3$) (two rotamers): δ 8.36 (br s, 1H), 7.60 (d, *J*=7 Hz, 1H), 7.48–7.33 (m, 10H), 7.23 (d, *J*=7 Hz, 1H), 5.21 (ma.) and 5.14 (mi.) (s, 4H), 4.75 (ma.) and 4.55 (mi.) (s, 2H), 4.18 (mi.) and 4.07 (ma.) (s, 2H), 3.69 (m, 2H), 3.72 (mi.) and 3.62 (ma.) (s, 3H), 3.61 (m, 2H), 1.31 (br s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 169.8 (mi.) and 169.4 (ma.), 167.7 (mi.) and 166.9 (ma.), 162.2, 155.7, 155.2, 152.8, 150.2, 136.2, 127.6–128.2, 95.2, 81.7, 67.5, 52.6 (mi.) and 52.2 (ma.), 49.7, 48.2, 47.1, 46.3, 27.9; mass spectrum ESI *m/z* 689.2511 ($C_{32}H_{38}N_6O_{10}$+Na requires 689.2547); IR (Nujol, cm^{-1}): 3250, 3055, 1754, 1670, 1266, 740–699.

4.1.3. Methyl-*N*-[2-(*N*$^{\alpha}$-benzyloxycarbonyl-*N*$^{\beta}$-*tert*-butoxycarbonyl-hydrazino)ethyl]-*N*-{[6-*N*-(benzyloxycarbonyl)adenin-9-yl]acetyl}glycinate (4). To a solution of **12** (151 mg, 0.46 mmol) and **8** (160 mg, 0.42 mmol) in dry DMF (1.9 mL), DhbtOH (75 mg, 0.46 mmol) and DIEA (81 μL, 0.46 mmol) were added. The mixture was cooled to 0 °C in an ice bath and then EDC·HCl (89 mg, 0.47 mmol) was added portionwise in 1 h. The mixture was allowed to warm to rt. The pH was brought to 8.5 with DIEA. The reaction was stirred for 21 h at rt. The solvent was removed in vacuo and the residue was partitioned between water (18 mL) and ethyl acetate (48 mL). The organic layer was washed with 0.1 M aq $KHSO_4$ (2×15 mL), water (2×15 mL), satd aq $NaHCO_3$ (2×30 mL) and brine (1×15 mL). All the aqueous phases were combined and extracted with ethyl acetate (2×100 mL). These organic phases were combined with the first and dried over Na_2SO_4. After filtration, the filtrate was concentrated to dryness to afford 271 mg of crude product as a pale yellow solid. Column chromatography on silica gel (ethyl acetate) afforded **4** (87 mg, R_f=0.35, 44%) as a white solid: mp=170 °C (dec); ^{1}H NMR (300 MHz, $CDCl_3$) (two rotamers): δ 9.61 (s, 1H), 8.70 (s, 1H), 8.32 (s, 1H), 8.04 (s, 1H), 7.42–7.29 (m, 10H), 5.27–5.19 (m, 4H), 5.05 (s, 2H), 4.13 (s, 2H), 3.79 (m, 2H), 3.70 (s, 3H), 3.69 (m, 2H), 1.40 (br s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 168.5, 165.5, 156.6, 156.2, 152.6, 150.5, 150.3, 148.9, 144.7, 135.1–134.3, 128.1–127.9, 120.7, 81.4, 67.7, 52.4, 53.0, 46.7, 43.5, 28.0; mass spectrum ESI *m/z* 713.2628 ($C_{33}H_{38}N_8O_9$+Na requires 713.26594; IR (Nujol, cm^{-1}): 3192, 1748, 1672, 1616, 1589, 1212, 1158, 1049, 1027, 997, 743, 698.

4.1.4. Methyl-*N*-[2-(*N*$^{\beta}$-benzyloxycarbonyl-*N*$^{\alpha}$-*tert*-butoxycarbonyl-hydrazino)ethyl]-*N*-[(thymin-1-yl)acetyl]-glycinate (5). To a solution of **10** (574 mg, 3.12 mmol) and **9** (1080 mg, 2.83 mmol) in dry DMF (7 mL), DhbtOH (508 mg, 3.11 mmol) and DIEA (532 μL, 3.12 mmol) were added. The mixture was cooled to 0 °C in an ice bath and then EDC·HCl (596 mg, 3.12 mmol) was added portionwise with vigorous stirring. The mixture was allowed to warm to rt. The pH was adjusted to basic conditions with DIEA. The reaction was stirred for 16 h. The solvent was removed in vacuo and the residue was partitioned between water (45 mL) and ethyl acetate (90 mL). The organic layer was washed with 0.1 M aq $KHSO_4$ (2×30 mL), water (2×30 mL), satd aq $NaHCO_3$ (2×30 mL) and brine (1×25 mL). The organic phase was dried over Na_2SO_4, filtered and the filtrate was concentrated in vacuo to afford 1.6 g of crude product as a pale yellow oil.

Column chromatography on silica gel (ethyl acetate/petroleum ether 9:1) afforded **5** (1.28 g, R_f=0.14, 82%) as a white solid: mp=120 °C (dec); ^{1}H NMR (300 MHz, $CDCl_3$) (mixture of rotamers): δ 8.99 (s, 1H), 7.37–7.27 (m, 5H), 7.07–6.98 (m, 1H), 5.17–5.14 (m, 2H), 4.6–3.9 (m, 4H), 3.79–3.65 (m, 7H), 1.90–1.86 (m, 3H), 1.47–1.39 (m, 9H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 169.5, 167.5, 164.2, 156.0, 155.0, 151.0, 141.3 and 140.7, 135.7, 129.0–127.1, 110.7, 81.0, 68.0, 52.3, 49.1, 47.7, 47.4, 46.0, 28.0, 12.2; mass spectrum ESI *m/z* 570.2149 ($C_{25}H_{33}N_5O_9$+Na requires 570.2173); IR (film, CH_2Cl_2, cm^{-1}): 3306, 2918, 1679, 1471, 1415, 1368, 1252, 1214, 1161, 1082, 1048, 1021, 756, 698.

4.1.5. Methyl-*N*-[2-(*N*$^{\beta}$-benzyloxycarbonyl-*N*$^{\alpha}$-*tert*-butoxycarbonyl-hydrazino)ethyl]-*N*-{[4-*N*-(benzyloxycarbonyl)cytosin-1-yl]acetyl}glycinate (6). To a solution of **11** (365.4 mg, 1.25 mmol) and **9** (434.5 mg, 1.14 mmol) in dry DMF (4 mL), DhbtOH (204.5 mg, 1.25 mmol) and DIEA (218 μL, 1.25 mmol) were added. The mixture was cooled to 0 °C in an ice bath and then EDC·HCl (239.6 mg, 1.25 mmol) was added portionwise with vigorous stirring. The mixture was allowed to warm to rt. The pH was adjusted to basic conditions with DIEA. The reaction was stirred overnight. The solvent was removed in vacuo and the residue was partitioned between water (17 mL) and ethyl acetate (36 mL). The organic layer was washed with 0.1 M aq $KHSO_4$ (2×12 mL), water (2×12 mL), satd aq $NaHCO_3$ (3×25 mL) and brine (1×12 mL). All the aqueous phases were combined and extracted with ethyl acetate (2×50 mL). These organic phases were combined with the first and dried over Na_2SO_4. After filtration, the filtrate was concentrated in vacuo to afford 943 mg of crude product as a pale yellow solid. Column chromatography on silica gel (gradient from ethyl acetate/petroleum ether 9:1 to pure ethyl acetate) afforded **6** (699 mg, R_f=0.17, 92%) as a white solid: mp=125–135 °C (dec); ^{1}H NMR (300 MHz, $CDCl_3$) (mixture of rotamers): δ 8.32 (br s, 1H), 7.61 (d, *J*=5 Hz, 1H), 7.33–7.23 (br m, 10H), 7.18 (d, *J*=5 Hz, 1H), 5.17–5.10 (two br s, 4H), 4.76–4.52 (m, 2H), 4.29–3.96 (m, 2H), 3.77–3.62 (m, 7H), 1.37 (br s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) (rotamers): δ 169.3, 167.1, 162.73, 156, 155.5, 152.3, 150.17 (mi.) and 149.64 (ma.), 135.02 (2C), 128.5–127.6, 94.8, 81.4, 67.6 (2C), 52.5 (mi.) and 52.0 (ma.), (some of the signals of the minor rotamer are obscured

by the major signals) 49.5, 49.2, 48.4, 47.7, 47.3, 47.0, 45.8, 28.0 (ma.) and 26.8 (mi.); mass spectrum ESI *m/z* 689.2536 ($C_{32}H_{38}N_6O_{10}$+Na requires 689.2547); IR (film, CH_2Cl_2, cm^{-1}): 3276, 1808, 1748, 1666, 1628, 1561, 1500, 1456, 1411, 1369, 1212, 1063, 789, 746, 699.

4.1.6. Methyl-*N*-[2-(N^{β}-benzyloxycarbonyl-N^{α}-*tert*-butoxycarbonyl-hydrazino)ethyl]-*N*-{[6-*N*-(benzyloxycarbonyl)adenin-9-yl]acetyl}glycinate (7). To a solution of **12** (159 mg, 0.48 mmol) and **9** (168 mg, 0.44 mmol) in dry DMF (2 mL), DhbtOH (79 mg, 0.48 mmol) and DIEA (85 μL, 0.49 mmol) were added. The mixture was cooled to 0 °C in an ice bath and then EDC·HCl (93 mg, 0.49 mmol) was added portionwise with vigorous stirring. The mixture was allowed to warm to rt. The pH was adjusted to basic conditions with DIEA (pH=9). The reaction was stirred for 48 h at rt. The solvent was removed in vacuo and the residue was partitioned between water (8 mL) and ethyl acetate (18 mL). The organic layer was washed with 0.1 M aq $KHSO_4$ (2×6 mL), water (2×6 mL), satd aq $NaHCO_3$ (4×12 mL) and brine (1×6 mL). All the aqueous phases were combined and extracted with ethyl acetate (2×30 mL). These organic phases were combined with the first and dried over Na_2SO_4. After filtration, the filtrate was dried in vacuo to afford 390 mg of crude product as a pale yellow solid. Column chromatography on silica gel (ethyl acetate) afforded **7** (212 mg, R_f=0.2, 70%) as a white solid: mp=114 °C (dec); ^{1}H NMR (300 MHz, $CDCl_3$) (many rotamers): δ 9.77 (br s, 0.8H), 9.32 (br s, 0.2H), 8.62–8.46 (m, 1H), 7.99–7.89 (m, 1H), 7.38–7.06 (m, 10H), 5.05–4.89 (m, 4H), 4.26–3.68 (m, 11H), 1.42 (br s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 150.8, 149.5, 135.7, 135.2, 128.6–127.8, 121.2, 110.7, 81.7, 68.2, 67.7, 52.9, 52.2, 49.3, 47.54, 46.9, 46.5, 45.8, 44.0, 43.2, 28.1, 27.0 (some signals are obscured); mass spectrum ESI *m/z* 713.2630 ($C_{33}H_{38}N_8O_9$+Na requires 713.2659); IR (film, CH_2Cl_2, cm^{-1}): 3250, 2977, 1747, 1672, 1615, 1589, 1541, 1466, 1412, 1368, 1321, 1287, 1212, 1157, 1083, 993, 895, 800, 738, 699, 642.

4.1.7. Methyl-*N*-[2-(N^{α}-benzyloxycarbonyl-N^{β}-*tert*-butoxycarbonyl-hydrazino)ethyl]glycinate (8). To a solution of glycine methyl ester hydrochloride (7.14 g, 57.1 mmol) in dry methanol (465 mL), DIEA (9.95 mL, 57.1 mmol) and **16** (16.00 g, 51.9 mmol) were added under stirring. Then a freshly prepared solution of $NaBH_3CN$ (3.91 g, 62.3 mmol) and $ZnCl_2$ (4.25 g, 31.2 mmol) in dry methanol (382 mL) was added dropwise to the reaction mixture. The mixture was stirred for 25 h while monitoring by TLC (eluent ethyl acetate/petroleum ether 7:3, R_f=0.20), and the solvent was removed in vacuo. The residue was dissolved in ethyl acetate and the organic phase was washed with satd aq $NaHCO_3$ and the aqueous layer was extracted four times with ethyl acetate. The combined organic phases were washed with brine and dried over Na_2SO_4. After filtration, the filtrate was concentrated in vacuo to afford 17.77 g of crude product as a yellow oil. Flash chromatography on silica gel (ethyl acetate/petroleum ether 8:2) afforded **8** (8.51 g, 43%) as a bright yellow oil; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.38–7.34 (m, 5H), 7.12 (br s, 1H), 5.18 (s, 2H), 3.73 (s, 3H), 3.61 (br t, 2H), 3.41 (s, 2H), 2.82 (t, *J*=5.9 Hz, 2H), 1.43 (br s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 172.6, 156.7, 155.3, 135.8, 128.4, 81.6, 67.4, 52.1, 48.7, 47.5, 46.1, 28.0; mass spectrum ESI *m/z* 382.1953 ($C_{18}H_{27}N_3O_6$+H requires 382.1978); IR (thin film, CCl_4, cm^{-1}): 3392, 2980, 1747, 1718, 1438, 1394, 1368, 1155, 1108, 1068.

4.1.8. Methyl-*N*-[2-(N^{β}-benzyloxycarbonyl-N^{α}-*tert*-butoxycarbonyl-hydrazino)ethyl]glycinate (9). A solution of **19** (610 mg, 1.98 mmol) and glycine methyl ester hydrochloride (207 mg, 1.65 mmol) in dry MeOH (18 mL) was stirred at 0 °C in an ice bath for 15 min. $NaBH_3CN$ (62 mg, 0.99 mmol) and AcOH (112 μL, 1.98 mmol) were added. The mixture was allowed to warm to rt and stirred for 3.5 h. The solvent was removed in vacuo and satd aq $NaHCO_3$ (20 mL) was added. The solution was extracted three times with ethyl acetate (3×60 mL). The combined organic phases were washed with brine, dried over Na_2SO_4 and concentrated in vacuo to afford 749 mg of crude product as a yellow oil. Column chromatography on silica gel (ethyl acetate/petroleum ether 8:2) afforded **9** (408 mg, R_f=0.2, 54%) as a pale yellow oil; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.34–7.26 (m, 5H), 5.15 (s, 2H), 3.70 (s, 3H), 3.64 (br t, *J*=5.1 Hz, 2H), 3.41 (s, 2H), 2.80 (br t, *J*=5.1 Hz, 2H), 1.40 (br s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 172.5, 156.3, 156.0, 135.7, 129.0–127.1, 81.0, 67.5, 51.4, 49.7, 49.0, 46.0, 27.8; mass spectrum ESI *m/z* 382.1955 ($C_{18}H_{27}N_3O_6$+H requires 382.1978); IR (neat film, cm^{-1}): 3317, 2977, 1736, 1455, 1412, 1367, 1218, 1152, 756, 699.

4.1.9. N^{β}-(*tert*-Butoxycarbonyl)-N^{β}-(2-hydroxyethyl)hydrazide (14). To a solution of **13** (5.00 g, 65.8 mmol) in dry ethanol (49 mL) at 0 °C, a solution of $(Boc)_2O$ (14.34 g, 65.8 mmol), previously dissolved in dry ethanol (39 mL), was added dropwise (30 min). The mixture was allowed to warm to rt and stirred for 26 h. The solvent was removed in vacuo to afford **14** (11.58 g, >98%) as a pale yellow oil; ^{1}H NMR (200 MHz, $CDCl_3$): δ 3.78 (t, *J*=4.9 Hz, 2H), 3.53 (t, *J*=4.9 Hz, 2H), 1.45 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 156.9, 80.6, 60.8, 51.7, 28.1; mass spectrum ESI *m/z* 199.1049 ($C_7H_{16}N_2O_3$+Na requires 199.1059); IR (neat film, cm^{-1}): 3335, 2977, 1690, 1402, 1368, 1252, 1170, 1059, 871, 769.

4.1.10. N^{α}-(Benzyloxycarbonyl)-N^{β}-(*tert*-butoxycarbonyl)-N^{β}-(2-hydroxyethyl)hydrazide (15). To a solution of NaOH (2.63 g, 65.8 mmol) in water (68 mL), CH_2Cl_2 (68 mL) was added and the mixture was cooled to 0 °C in an ice bath. Then **14** (11.58 g, 65.8 mmol) was added. Cbz-Cl (9.3 mL, 65.8 mmol) was added dropwise. The mixture was allowed to warm to rt and stirred for 20 h. After this time, the stirring was stopped, and the organic layer was collected and washed with water and a 20% citric acid aq solution. The organic layer was dried over Na_2SO_4 and filtered. The filtrate was collected and the solvent was removed in vacuo to afford **15** (19.01 g, 93%) as a pale yellow oil; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.36–7.32 (m, 5H), 6.90 (br s, 1H), 5.18 (s, 2H), 3.70 (t, *J*=4.4 Hz, 2H), 3.58 (t, *J*=4.4 Hz, 2H), 1.42 (br s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 157.8, 155.2, 135.4, 129.2–127.7, 81.6, 67.6, 59.2, 53.0, 27.9; mass spectrum ESI *m/z* 333.1418 ($C_{15}H_{22}N_2O_5$+Na requires 333.1426); IR (neat film, cm^{-1}): 3294, 2978, 1713, 1499, 1456, 1396, 1369, 1256, 1216, 1164, 1066, 864, 753, 699.

4.1.11. N^{α}-(Benzyloxycarbonyl)-N^{β}-(*tert*-butoxycarbonyl)-N^{β}-(formylmethyl)hydrazide (16). To a solution of **15** (19.00 g, 61.3 mmol) in water-saturated CH_2Cl_2

(530 mL), Dess–Martin periodinane (54.60 g, 129 mmol) was added under stirring. The white suspension was stirred for 50 min at rt. Then, methyl *tert*-butyl ether (MTBE) (190 mL) and a satd aq $NaHCO_3$ (190 mL) containing $Na_2S_2O_3$ (106.5 g, 674 mmol) were added to the mixture, and stirred vigorously for 25 min. The stirring was stopped and the two layers were separated. The aqueous layer was extracted twice with CH_2Cl_2 (240 mL). The combined organic phases were washed with satd aq $NaHCO_3$ (60 mL), water (2×80 mL) and brine (2×60 mL). The organic layer was dried over Na_2SO_4 and concentrated to dryness to afford **16** (17.00 g, R_f=0.28 in hexane/ethyl acetate 1:1, 90%) as a yellow oil. The product obtained in this way was usually sufficiently pure to be used in the next step. Pure product and an analytically pure sample were obtained by column chromatography on silica gel (gradient from hexane/ethyl acetate 6:4 to hexane/ethyl acetate 1:1); ^{1}H NMR (300 MHz, $CDCl_3$): δ 9.67 (br s, 1H), 7.35 (m, 5H), 6.84 (s, 1H), 5.17 (s, 2H), 4.30 (br s, 2H), 1.42 (br s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 197.8, 157.8, 155.2, 135.4, 128.5–128.2, 81.6, 67.7, 59.5, 27.9; mass spectrum ESI *m/z* 331.1263 ($C_{15}H_{20}N_2O_5$+Na requires 331.1270); IR (neat film, cm^{-1}): 3305, 2979, 1732, 1500, 1456, 1370, 1260, 1221, 1152, 1067, 1029, 989, 904, 855, 745, 699.

4.1.12. N^{β}-(Benzyloxycarbonyl)-N^{β}-(2-hydroxyethyl)hydrazide (17). To a solution of **13** (6.37 g, 90% by weight, 75 mmol) in CH_2Cl_2 (100 mL) at 0 °C, Et_3N (10.4 mL, 75 mmol) was added. Under vigorous stirring, Cbz-Cl (10.6 mL, 75 mmol) was added dropwise. The resulting mixture was allowed to warm to rt and then stirred for 24 h. The mixture was concentrated in vacuo to give 22.70 g of a pale yellow oil. The crude product was suspended in ethyl acetate and filtered on a 3 cm silica gel bed, isolating the first fraction containing the unreacted Cbz-Cl and eluting the product with ethyl acetate afforded **17** (12.52 g, R_f=0.19, 80%) as a colourless oil; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.4–7.3 (m, 5H), 5.15 (s, 2H), 4.19 (br s, 2H), 3.80 (t, J=4.5 Hz, 2H), 3.60 (t, J=4.5 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 157.5, 136.0, 128.3–126.9, 67.5, 60.3, 51.9; mass spectrum ESI *m/z* 233.0891 ($C_{10}H_{14}N_2O_3$+Na requires 233.0902); IR (neat film, cm^{-1}): 3412, 3344, 3055, 2948, 1696, 1626, 1455, 1416, 1359, 1266, 1217, 1126, 1060, 910, 735, 700.

4.1.13. N^{β}-(Benzyloxycarbonyl)-N^{α}-(*tert*-butoxycarbonyl)-N^{β}-(2-hydroxyethyl)hydrazide (18). To a solution of **17** (1.025 g, 4.88 mmol) in dry THF (15 mL), Et_3N (680 μL, 4.9 mmol) was added dropwise and the mixture was cooled to 0 °C in an ice bath. $(Boc)_2O$ was added under vigorous stirring and the mixture was allowed to warm to rt. After stirring for 3 h, the mixture was concentrated to dryness. The residue was partitioned between water and ethyl acetate. The aqueous layer was extracted three times with ethyl acetate. The combined organic phases were dried over Na_2SO_4 and concentrated to dryness to afford 1.46 g of the crude product as a pale yellow oil. Column chromatography on silica gel (ethyl acetate/petroleum ether 1:1) afforded **18** (1.08 g, R_f=0.22, 71.3%) as a pale yellow oil; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.33–7.28 (m, 5H), 6.50 (br s, 1H), 5.16 (s, 2H), 3.70 (br s, 2H), 3.60 (br s, 2H), 1.42 (br s, 9H); ^{13}C NMR ($CDCl_3$): δ 156.3, 156.0, 135.6, 129.1–127.1, 82.0, 67.9, 58.8, 52.8, 27.9; mass spectrum ESI *m/z* 333.1419 ($C_{15}H_{22}N_2O_5$+Na requires 333.1426); IR (neat film, cm^{-1}): 3299, 2978, 1713, 1499, 1455, 1413, 1368, 1288, 1254, 1214, 1162, 1065, 755, 698.

4.1.14. N^{β}-(Benzyloxycarbonyl)-N^{α}-(*tert*-butoxycarbonyl)-N^{β}-(formylmethyl)hydrazide (19). To a solution of **18** (1.06 g, 3.42 mmol) in water-saturated CH_2Cl_2 (22 mL), Dess–Martin periodinane (3.05 g, 7.19 mmol) was added under stirring. The white suspension was stirred for 2.5 h at rt. Then, methyl *tert*-butyl ether (MTBE) (11 mL) and a satd aq $NaHCO_3$ (11 mL) containing $Na_2S_2O_3$ (5.95 g, 37.6 mmol) were added to the mixture and stirred vigorously for 30 min. The stirring was stopped and the two layers were separated. The aqueous layer was extracted three times with CH_2Cl_2. The combined organic phases were washed with satd aq $NaHCO_3$, twice with water and twice with brine. The organic phase was dried over Na_2SO_4 and concentrated in vacuo to afford 1.02 g of crude product as a yellow oil. Column chromatography on silica gel (gradient from petroleum ether/ethyl acetate 6:4 to pure ethyl acetate) afforded **19** (933 mg, 88%, R_f=0.33 in petroleum ether/ethyl acetate 1:1) as a pale yellow oil; ^{1}H NMR (300 MHz, $CDCl_3$): δ 9.68 (br s, 1H), 7.34–7.26 (m, 5H), 5.17 (s, 2H), 4.34 (br s, 2H), 1.42 (br s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 191.4, 156.3, 156.0, 135.0, 129.0–127.0, 82.0, 68.3, 55.2, 28.0; mass spectrum EI *m/z* 308 $(M)^+$. IR (neat film, cm^{-1}): 3307, 2980, 1713, 1497, 1456, 1412, 1369, 1255, 1157, 1069, 1031, 738, 699.

4.1.15. Methyl-*N*-[-2-[2-(N^{α}-benzyloxycarbonyl-N^{β}-*tert*-butoxycarbonyl-hydrazino)ethyl]-*N*-[(thymin-1-yl)acetyl]glycyl]aminoethyl-*N*-[(thymin-1-yl)acetyl]glycinate ([N^{α}-Cbz-N^{β}-Boc-*hyd*(T)PNA]-[*aeg*(T)PNA-COOCH$_3$], 20). Compound **23** (100 mg, 0.19 mmol) was dissolved in dry DMF (2.0 mL) and a solution of pentafluorophenol (38 mg, 0.21 mmol) in dry DMF (1 mL) was added. The solution was cooled to 0 °C in an ice bath. DCC (47 mg, 0.23 mmol) was added under vigorous stirring. The solution was stirred for 1 h at 0 °C and, then, for 5 h at rt. The mixture was filtered in an inert atmosphere to remove the DCU and the white solid was washed twice with dry DMF (2×1 mL). The filtrate was collected. In another round-bottomed flask, **29** (77 mg, 0.19 mmol) was dissolved in dry DMF (4 mL) and DIEA (53 μL, 0.31 mmol), and stirred for 1 min. This mixture was then added to the previous filtrate containing the pentafluorophenol ester and stirred for 24 h. The solvent was concentrated in vacuo to obtain 276 mg of crude product. Column chromatography on silica gel (ethyl acetate/methanol 8:2) afforded **20** (139 mg, R_f=0.13, 91%) as a white solid, no melting was observed; at 160 °C, it became a yellow rubber (dec); ^{1}H NMR (300 MHz, DMSO-d_6) (mixture of rotamers): δ 8.30 (m, 2H), 7.39–7.23 (m, 7H), 5.11 (s, 2H), 4.68–4.06 (m, 12H), 4.02–3.86 (m, 2H), 3.71–3.43 (m, 5H), 1.70–1.67 (m, 6H), 1.36–1.32 (m, 9H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 170.0, 169.5, 168.5, 167.8, 164.3 (2C q. ov.), 156.3, 156.0, 151.0 (2 q. ov.), 142.0 (2 =CH ov.), 136.3, 128.8–128.3 (5CH arom. ov.), 108.8 (2 =C q. ov.), 79.7, 65.7, 51.7, 49.5–46.7 (7CH_2 ov.), 38.0, 27.4, 11.7 (2CH_3 ov.); mass spectrum ESI *m/z* 836.3192 ($C_{36}H_{47}N_9O_{13}$+Na requires 836.3191); IR (Nujol, cm^{-1}): 3481, 1674, 1541, 1337, 1208, 1136, 1082, 1048, 1021, 837, 797, 719.

4.1.16. [Boc-*aeg*(T)PNA] [N^{β}-Cbz-*hyd*(T)PNA-COOCH$_3$] (21). Compound **32** (301 mg, 0.78 mmol) was dissolved in dry DMF (2 mL) and then **27** (400 mg, 0.71 mmol) was added. DhbtOH (128 mg, 0.78 mmol) and DIEA (268 μL, 202 mg, 1.57 mmol) were added under vigorous stirring. The reaction mixture was then cooled to 0 °C in an ice bath, and EDC·HCl (150 mg, 0.78 mmol) was added portionwise. The mixture was allowed to warm to rt and the pH was adjusted to 9 with DIEA. The mixture was stirred for 85 h. The reaction mixture was then concentrated to dryness, and dissolved in water (20 mL) and ethyl acetate (30 mL). The aqueous layer was extracted with ethyl acetate (4×15 mL). The organic phases were collected and washed with 0.1 M aq $KHSO_4$ (2×80 mL), water (2×60 mL), satd aq $NaHCO_3$ (3×60 mL) and brine (1×60 mL). All of the aqueous phases were combined and extracted with ethyl acetate (2×80 mL). These organic phases were combined with the first and dried over Na_2SO_4. The organic phase was filtered and concentrated to dryness in vacuo to afford 426 mg of the crude product. Column chromatography on silica gel (ethyl acetate/MeOH 8:2) afforded **21** (376 mg, R_f=0.29, 65%) as a white solid: mp=95–100 °C (dec); ^{1}H NMR (300 MHz, $CDCl_3$) (mixture of rotamers): δ 7.40–7.24 (m, 5H), 7.05–6.95 (m, 2H), 5.22–5.07 (m, 2H), 4.52–3.77 (m, 8H), 3.73–3.13 (m, 11H), 1.85–1.82 (m, 6H), 1.42 (br s, 9H); ^{13}C NMR (50 MHz, $CDCl_3$) (some rotamers not completely attributed are present): δ 169.7, 168.1–167.2 (3C q. ov.), 165.6–156.3 (4C q. ov.), 151.5 (2C q. ov.), 141.6–141.2 (2 =CH ov.), 135.6, 128.4 (5CH arom. ov.), 110.6 (2 =C q. ov.), 79.8, 68.3, 53.4 (mi.) and 52.3 (ma.), 49.5–46.6 ($7CH_2$ ov.), 38.5, 28.4, 12.2 ($2CH_3$ ov.); mass spectrum ESI *m/z* 836.3181 ($C_{36}H_{47}F_3N_9O_{13}$+Na requires 836.3185); IR (Nujol, cm^{-1}): 3416, 1712, 1505, 1263, 1166, 721.

4.1.17. [Boc-*aeg*(T)PNA] [N^{α}-Cbz-*hyd*(T)PNA-COOCH$_3$] (22). Compounds **32** (230 mg, 0.60 mmol) and **25** (304 mg, 0.54 mmol) were dissolved in dry DMF (1.5 mL) with stirring. DhbtOH (97 mg, 0.60 mmol) and DIEA (204 μL, 154 mg, 1.19 mmol) were added. The mixture was then cooled to 0 °C in an ice bath. EDC·HCl (114 mg, 0.60 mmol) was added portionwise. The reaction was allowed to warm to rt and the pH was adjusted to 9 with DIEA (140 μL). The mixture was stirred for 24 h and then concentrated to dryness in vacuo. The residue was dissolved in water (20 mL) and ethyl acetate (30 mL). The organic layer was washed with 0.1 M aq $KHSO_4$ (2×15 mL), water (2×15 mL), satd aq $NaHCO_3$ (2×20 mL) and brine (2×15 mL). All of the aqueous phases were combined and extracted with ethyl acetate (3×20 mL). These organic phases were combined with the first and dried over Na_2SO_4. The organic phase was filtered and concentrated to dryness to afford 324 mg of the crude product as a yellow solid. Column chromatography on silica gel (ethyl acetate/MeOH 8:2) afforded **22** (254 mg, R_f=0.28, 56%) as a white solid: mp=100–120 °C (dec); ^{1}H NMR (300 MHz, $CDCl_3$) (mixture of rotamers): δ 7.36–7.32 (m, 5H), 7.14–6.90 (m, 2H), 5.18 (ma.) and 5.17 (mi.) (m, 2H), 4.51–3.80 (m, 8H), 3.80–3.00 (m, 11H), 1.92–1.83 (m, 6H), 1.42 (br s, 9H); ^{13}C NMR (50 MHz, $CDCl_3$) (some rotamers not completely assigned are present): δ 171.4, 169.6–167.3 (3C q. ov.), 165.5–164.7 (4C q. ov.), 151.5 (2C q. ov.), 143.1–139.5 (2 =CH ov.), 135.5, 130.1–126.9 (5CH arom. ov.), 110.6 (2 =C q. ov.), 79.9, 67.7, 55.1, 51.0–46.5 ($7CH_2$ ov.), 38.2, 28.3, 13.4–12.1 ($2CH_3$ ov.); mass spectrum ESI *m/z* 836.3169 ($C_{36}H_{47}F_3N_9O_{13}$+Na requires 836.3185); IR (KBr, cm^{-1}): 3467, 3066, 1666, 1473, 1421, 1385, 1251, 1218, 1172, 1092, 1044, 964, 819, 784, 734, 698, 564, 462.

4.1.18. *N*-[2-(N^{α}-Benzyloxycarbonyl-N^{β}-*tert*-butoxycarbonyl-hydrazino)ethyl]-*N*-[(thymin-1-yl)acetyl]glycine (23). Compound **2** (1.00 g, 1.83 mmol) was dissolved in 2 M KOH aq (30 mL) and the solution was stirred for 1 h at rt. The pH was then adjusted to 2–3 with 1 M aq HCl. A white precipitate was observed. The mixture was extracted with CH_2Cl_2 (3×70 mL), and the organic phases were combined and dried over Na_2SO_4, filtered and the filtrate concentrated to dryness to afford **23** (927 mg, 95%) as a white solid: mp=147 °C (dec); ^{1}H NMR (300 MHz, $CDCl_3$) (two rotamers): δ 9.80 (br s, 1H), 9.30 (br s, 1H), 7.39–7.28 (m, 5H), 7.08 (ma.) and 7.05 (mi.) (s, 1H), 5.18 (s, 2H), 4.15 (s, 2H), 4.11 (s, 2H), 3.66 (m, 2H), 3.63 (m, 2H), 1.92 (s, 3H), 1.37 (br s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.6, 168.3, 167.8, 164.5, 155.1, 151.4, 141.7, 135.7, 128.4, 110.5, 81.5, 67.5, 48.2, 47.9, 47.5, 46.2, 27.9, 12.0; mass spectrum FAB *m/z* 556 (M+Na)$^+$; IR (Nujol, cm^{-1}): 3515, 3263, 1714, 1694, 1669, 1412, 1252, 1215, 1163, 1082, 1045, 1025, 963, 906, 841, 783, 756, 736, 698.

4.1.19. *N*-[2-(N^{β}-Benzyloxycarbonyl-N^{α}-*tert*-butoxycarbonyl-hydrazino)ethyl]-*N*-[(thymin-1-yl)acetyl]glycine (24). Compound **5** (200 mg, 0.37 mmol) was dissolved in 2 M aq KOH (10 mL) and the solution was stirred for 1 h at rt. The pH was then adjusted to 2–3 with 1 M aq HCl. A white precipitate was observed. The mixture was extracted with CH_2Cl_2 (3×20 mL), and the organic phases were combined and dried over Na_2SO_4, filtered and the filtrate concentrated to dryness to afford **24** (185 mg, 95%) as a white solid: mp=103–110 °C (dec); ^{1}H NMR (300 MHz, $CDCl_3$) (two rotamers): δ 8.99 (br s, 1H), 7.36 (m, 5H), 7.07 (ma.) and 7.02 (mi.) (s, 1H), 5.14 (s, 2H), 4.49 (s, 2H), 4.13 (s, 2H), 3.89 (m, 2H), 3.60 (m, 2H), 1.85 (s, 3H), 1.44 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 172.2, 167.5, 164.2, 156.3, 156.0, 151.0, 141.0 and 142.0, 135.9, 128.1–129, 110.7, 81.0, 68.1, 49.0, 48.5, 48.4, 46.2, 28.3, 12.3; mass spectrum ESI *m/z* 556 (M+Na)$^+$; IR (thin film, CH_2Cl_2, cm^{-1}): 3474, 3264, 2614, 1694, 1681, 1471, 1368, 1286, 1217, 1085, 1049, 1021, 967, 787, 756.

4.1.20. Methyl-*N*-[2-(N^{α}-benzyloxycarbonyl-hydrazino)ethyl]-*N*-[(thymin-1-yl)acetyl]glycinate trifluoroacetic salt (25). Compound **2** (1.70 g, 3.11 mmol) was dissolved in a solution of TFA (7.09 g, 4.8 mL, 62.16 mmol) at 40% by weight in CH_2Cl_2 (8 mL) under vigorous stirring. Gas formation was observed. The reaction was stirred overnight. The solution was concentrated in vacuo to an orange oil. The oil was stirred in diethyl ether for 1 h at rt and a white precipitate was obtained. Centrifugation afforded **25** (1.35 g, 77%) as a white solid: mp=115 °C (dec); ^{1}H NMR (300 MHz, DMSO-d_6) (mixture of rotamers): δ 9.90 (br s, 1H), 7.38–7.33 (m, 6H), 5.13 (ma.) and 5.09 (mi.) (s, 2H), 4.69 (mi.) and 4.67 (ma.) (m, 2H), 4.50–4.25 (m, 2H), 4.13 (m, 2H), 3.72 (m, 2H), 3.62 (br s, 3H), 1.76 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) (mixture of rotamers): δ 170.0, 167.8, 164.8, 164.5, 151.4, 142.4, 135.5, 128.8–128.2, 108.5, 66.1, 49.5, 48.2, 47.1, 46.6, 46.0, 12.2; mass

spectrum ESI *m/z* 448 ($M-CF_3COOH+H)^+$; IR (Nujol, cm^{-1}): 3200, 1675, 1216.

4.1.21. Methyl-*N*-[2-(N^{α}-benzyloxycarbonyl-hydrazino)ethyl]-*N*-{[4-*N*-(benzyloxycarbonyl)cytosin-1-yl]-acetyl}glycinate trifluoroacetic salt (26). Compound **3** (100 mg, 0.15 mmol) was dissolved in a solution of TFA (342 mg, 220 μL, 3.0 mmol) at 20% by weight in CH_2Cl_2 (1.5 mL) under vigorous stirring. Gas formation was observed. The reaction was stirred overnight. The solution was concentrated in vacuo to an orange oil. The oil was dissolved in CH_2Cl_2 and concentrated in vacuo. This operation was repeated several times in order to obtain a bright yellow foam. Column chromatography on silica gel (ethyl acetate/MeOH 9:1) afforded **26** (70 mg, 69%) as a white solid: mp=110 °C (dec); ^{1}H NMR (300 MHz, CD_3OD) (many rotamers): δ 7.91 (br s, 1H), 7.41–7.31 (m, 10H), 7.13 (br s, 1H), 5.22 (mi.) and 5.15 (ma.) (s, 4H), 4.69 (mi.) and 4.67 (ma.) (m, 2H), 4.50–4.25 (m, 2H), 4.13 (m, 2H), 3.72 (m, 2H), 3.62 (br s, 3H); ^{13}C NMR (75 MHz, CD_3OD) (many rotamers): δ 170.0, 169.1, 167.8, 163.0, 162.8, 152.7, 151.5, 151.2, 135.9, 135.3, 128.2–127.5 95.2, 95.2, 67.7, 67.2, 52.1, 49.3, 47.3, 46.7, 46.2, 45.5, 43.9, 42.0; mass spectrum ESI *m/z* 589.2016 ($C_{27}H_{30}N_6O_8$+Na requires 589.2023)$^+$; IR (Nujol, cm^{-1}): 3474, 1731, 1663, 1640, 1456, 1374, 1201, 1140, 1028, 909, 838, 800, 722, 698.

4.1.22. Methyl-*N*-[2-(N^{β}-benzyloxycarbonyl-hydrazino)ethyl]-*N*-[(thymin-1-yl)acetyl]glycinate trifluoroacetic salt (27). Compound **5** (100 mg, 0.18 mmol) was dissolved in a solution of TFA (416 mg, 270 μL, 3.65 mmol) at 40% by weight in CH_2Cl_2 (470 μL). Gas formation was observed. The reaction was stirred for 16 h at rt. The solution was concentrated in vacuo. The solid was washed three times with Et_2O and separated by centrifugation. The residue was dried in vacuo to afford **27** (83 mg, 80%) as a white solid: mp=130 °C (dec); ^{1}H NMR (300 MHz, $CDCl_3$) (two rotamers): δ 8.99 (m, 1H), 7.35–6.75 (m, 6H), 5.15 (s, 2H), 4.75–3.50 (m, 11H), 1.95 (ma.) and 1.90 (mi.) (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 172.0, 164.6, 164.2, 156.0, 151.0, 141.5 and 141.0, 135.5, 129.0–127.7, 110.8, 68.0, 52.3, 49.1, 48.1, 47.3, 46.6, 12.1; mass spectrum ESI *m/z* 448.3 ($M-CF_3COOH+H)^+$; IR (film, CH_2Cl_2, cm^{-1}): 3208, 3037, 2962, 2921, 2846, 1745, 1680, 1470, 1439, 1419, 1358, 1260, 1212, 1167, 1085, 1020, 967, 906, 799, 756, 739, 701.

4.1.23. Methyl-*N*-[2-(N^{β}-benzyloxycarbonyl-hydrazino)ethyl]-*N*-{[4-*N*-(benzyloxycarbonyl)cytosin-1-yl]-acetyl}glycinate trifluoroacetic salt (28). Compound **6** (100 mg, 0.15 mmol) was dissolved in a solution of TFA (1.026 g, 670 μL, 3.00 mmol) at 20% by weight in CH_2Cl_2 (3.1 mL) under vigorous stirring. Gas formation was observed. The reaction was stirred overnight. The solution was concentrated in vacuo to a pale yellow oil. The oil was dissolved in few drops of CH_2Cl_2 and treated with petroleum ether. A white precipitate was observed. The precipitate was dried in vacuo to afford 102 mg of crude product as a pale yellow solid foam. Column chromatography on silica gel (ethyl acetate/methanol 9:1) afforded **28** (70 mg, 69%) as a white solid: no melting was observed; at 120 °C, it became a brown rubber (dec); ^{1}H NMR (300 MHz, CD_3OD) (two rotamers): δ 7.74 (br d, 1H), 7.42–7.29 (m, 10H), 7.24 (br d, 1H), 5.23–5.16 (m, 4H), 4.59–4.00 (m, 6H), 3.78–3.66 (m, 5H); ^{13}C NMR (75 MHz, CD_3OD): δ 169.4, 165.2, 164.7, 159.1, 158.4, 154.3, 151.8, 148.4, 137.1, 129.6–128.7, 96.9 (ma.) and 95.9 (mi.), 69.3, 68.7, 52.8, 51.9, 47.8, 47.2, 46.5; mass spectrum ESI *m/z* 589.2016 ($C_{29}H_{31}F_3N_6O_{10}$-CF_3COOH+Na requires 589.2023); IR (Nujol, cm^{-1}) 3341, 1754, 1675, 1566, 1504, 1454, 1208, 1128, 841, 800, 722, 699.

4.1.24. *N*-[-2-[2-(N^{α}-Benzyloxycarbonil-N^{β}-*tert*-butoxycarbonyl-hydrazino)ethyl]-*N*-[(thymin-1-yl)acetyl]glycyl]aminoethyl-*N*-[(thymin-1-yl)acetyl]glycine ([N^{α}-Cbz-N^{β}-Boc-*hyd*(T)PNA] [*aeg*(T)PNA-COOH], 30). Compound **20** (132 mg, 0.16 mmol) was dissolved in methanol (7 mL), and a solution of LiOH (13 mg, 0.31 mmol) in methanol (2.5 mL) was added. The mixture was stirred for 24 h and then another portion of LiOH (13 mg, 0.31 mmol) in methanol (1 mL) was added. After a further 5 h, the solvent was concentrated in vacuo. The residue was dissolved in water (a few drops) and 1 M aq $KHSO_4$ was added dropwise to adjust the pH to 2. The solution was extracted 10 times with ethyl acetate, and the solvent was concentrated in vacuo to afford **30** (65 mg, 50%) as a white solid: no melting was observed; at 180 °C, it became brown; ^{1}H NMR (300 MHz, CD_3OD): δ 7.38–7.33 (m, 7H), 5.18 (ma.) and 5.16 (mi) (s, 2H), 4.70–3.95 (m, 8H), 3.75–3.40 (m, 8H), 1.85 (m, 6H), 1.40 (br s, 9H); ^{13}C NMR (75 MHz, CD_3OD): δ 173.4, 167.4–156.0 (7C q. ov.), 152.0 (2C q. ov.), 144.4, 143.6, 134.1, 129.5–129.1 (5CH arom. ov.), 111.0 (2C q. ov.), 83.1, 68.4, 50.0–46.7 ($8CH_2$ ov.), 28.2 ($3CH_3$ ov.), 11.8 ($2CH_3$ ov.); mass spectrum ESI *m/z* 844.2871 ($C_{35}H_{45}N_9O_{13}$-H+2Na requires 844.2854); IR (Nujol, cm^{-1}): 3436, 1675, 1256, 1205, 1141.

4.1.25. Methyl-*N*-[-2-[2-(N^{α}-benzyloxycarbonyl-hydrazino)ethyl]-*N*-[(thymin-1-yl)acetyl]glycyl]aminoethyl-*N*-[(thymin-1-yl)acetyl]glycinate trifluoroacetic salt ([N^{α}-Cbz-*hyd*(T)PNA] [*aeg*(T)PNA-$COOCH_3$] trifluoroacetic salt, 31). Compound **20** (143 mg, 0.18 mmol) was dissolved in a freshly prepared solution of trifluoroacetic acid (270 μL, 3.52 mmol) at 20% by weight in CH_2Cl_2 (1.2 mL). The mixture was stirred overnight. The solvent was evaporated at reduced pressure to afford a pale yellow oil. The residue was repeatedly diluted with CH_2Cl_2 and concentrated until **31** was obtained (143 mg, >98%) as a white foam: mp=190 °C (dec); ^{1}H NMR (300 MHz, CD_3OD) (mixture of rotamers): δ 7.45–7.17 (m, 7H), 5.37–4.87 (m, 4H), 4.72–4.10 (m, 4H), 3.99–3.19 (m, 11H), 1.84–1.76 (m, 6H); ^{13}C NMR (75 MHz, CD_3OD) (some not completely attributed rotamers): δ 172.2, 172.0, 169.8 (2C q. ov.), 166.9 (2C q. ov.), 159.3, 153.1 (2C q. ov.), 144.2–143.8 (2 =CH ov.), 137.9, 137.4, 129.6–128.8 (5CH arom. ov.), 111.1–110.7 (2C q. ov.), 68.6 (mi.) and 68.0 (ma.), 53.3, 50.0–47.3 ($7CH_2$ ov.), 38.0, 12.3 ($2CH_3$ ov.); mass spectrum ESI *m/z* 736.2653 ($C_{33}H_{40}F_3N_9O_{13}$-CH_3COOH+Na requires 736.2667); IR (Nujol, cm^{-1}): 3433, 1674, 1201, 1140, 1025, 797, 719.

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