



## Carboxyalkyl peptoid PNAs: synthesis and hybridization properties

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### ABSTRACT

*N*<sup>γ</sup>-Carboxyalkyl modified peptide nucleic acids (PNAs), containing the four canonical nucleobases, were prepared via solid-phase oligomerization. The inserted peptoid monomers **1** and **2** were constructed through simple synthetic procedures, utilizing appropriate glycidol and iodoalkyl electrophiles. Thermal denaturation studies, performed with complementary antiparallel DNA strands, demonstrated that the length of the *N*<sup>γ</sup>-side chain strongly influences the modified PNAs hybridization properties. Moreover, multiple negative charges on the oligoamide backbone, when present on  $\gamma$ -nitrogen C<sub>6</sub> side chains proved to be beneficial for the oligomers' water solubility and DNA hybridization specificity.

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### 1. Introduction

Nucleic acids encode structures and functions of all living systems. None of the multitudinous synthetically available homomorphous frameworks has ever displayed the phospho(deoxy)ribosyl backbone properties.<sup>1</sup> However, polyamides bearing canonical nucleobases, such as *N*-(2-aminoethyl)glycine PNA (aegPNA),<sup>2</sup> deeply interfere with the DNA/RNA functions and, in 20 years of biophysical and biological studies, have demonstrated unmatched recognition and antisense properties.<sup>3</sup>

The considerable biological stability, the excellent nucleic acids binding properties, and the appreciable chemical simplicity, make PNA an invaluable tool in molecular biology.<sup>4</sup> Unfortunately, despite these remarkable properties, PNA has two serious limitations: low water solubility<sup>5</sup> and poor cellular uptake.<sup>6</sup> Considerable efforts have been made to circumvent these drawbacks, and a conspicuous number of new analogs have been proposed,<sup>7</sup> including those with the  $\gamma$ -nitrogen modified *N*-(2-aminoethyl)-glycine (aeg) units.<sup>8</sup>

In an elegant contribution by the Nielsen group,<sup>9</sup> an accurate investigation of the *N*<sup>γ</sup>-methylated PNA hybridization properties was reported. In this study it was found that the formation of PNA/DNA (or RNA) duplexes was not altered in case of a 30% *N*<sup>γ</sup>-methyl nucleobase substitution. However, the hybridization efficiency per *N*-methyl unit in a PNA, decreased on increasing the *N*-methyl content.

The negative impact of the  $\gamma$ -N alteration reported by Nielsen, did not discourage further investigations. The potentially informative

triazine-tagged oligoglycines systems,<sup>10</sup> the oligomeric thymine-functionalized peptoids,<sup>8d</sup> the achiral *N*<sup>γ</sup>- $\omega$ -aminoalkyl nucleic acids,<sup>8a</sup> constitute convincing examples of  $\gamma$ -nitrogen beneficial modification. In particular, the Liu group contribution,<sup>8a</sup> revealed an unexpected stereoelectronic effect played by the *N*<sup>γ</sup>-side chain length. In their stringent analysis it was demonstrated that while short  $\omega$ -amino *N*<sup>γ</sup>-side chains negatively influenced the modified PNAs hybridization properties, longer  $\omega$ -amino *N*<sup>γ</sup>-side chains positively modulated nucleic acids binding. It was also found that suppression of the positive  $\omega$ -aminoalkyl charge (i.e., through acetylation) caused no reduction in the hybridization affinity, suggesting that factors different from mere electrostatic stabilizing interactions were at play in the hybrid amino-peptoid-PNA/DNA (RNA) duplexes.<sup>11</sup>

Considering the interesting results achieved in the case of *N*-(2-alkylaminoethyl)-glycine units,<sup>8,9</sup> and on the basis of poor hybridization properties showed by two fully peptoidic homopyrimidine oligomers synthesized by our group,<sup>8b</sup> we decided to explore the effects of anionic residues at the  $\gamma$ -nitrogen in a PNA framework on the in vitro hybridization properties.

The *N*-(carboxymethyl) and the *N*-(carboxypentamethylene) *N*<sup>γ</sup>-residues, present in monomers **1** and **2** (Fig. 1) were chosen in order to evaluate possible side chains length-dependent thermal denaturations effects, and with the aim to respond to the water-solubility issue, which is crucial for the specific subcellular distribution.<sup>6a</sup>

The synthesis of a negatively charged *N*-(2-carboxyalkylaminoethyl)-glycine backbone (negative charged PNA are rarely found in literature)<sup>12</sup> was based on the idea of taking advantage of the availability of a multitude of efficient methods for the gene

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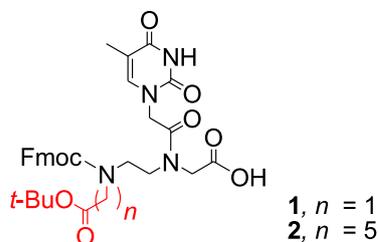


Fig. 1. Structures of bis-protected thyminylated  $N^{\omega}$ -carboxyalkyl monomers **1** and **2**.

cellular delivery based on the interaction of carriers with negatively charged groups. Most of the nonviral gene delivery systems are, in fact, based on cationic lipids<sup>13</sup> or cationic polymers<sup>14</sup> interacting with negative charged genetic vectors. Furthermore, the neutral backbone of PNA prevents them being recognized by proteins, which interact with DNA, and PNA/DNA chimeras should be synthesized for applications such as transcription factors scavenging (decoy)<sup>15</sup> or activation of RNA degradation by RNase-H (as in antisense drugs).<sup>3d</sup> This lack of recognition is partly due to the lack of negatively charged groups and of the corresponding electrostatic interactions with the protein counterpart.<sup>16</sup>

In the present work we report the synthesis of the bis-protected thyminylated  $N^{\omega}$ -carboxyalkyl monomers **1** and **2** (Fig. 1), the solid-phase oligomerization and the base-pairing behavior of four oligomeric peptoid sequences **3–6** (Fig. 2) incorporating, to various extents and at different positions, the monomers **1** and **2**.

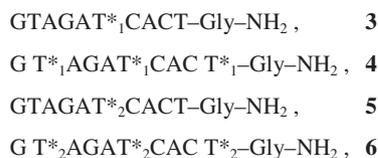


Fig. 2. Structures of target oligomers **3–6**. T\* represents the modified thyminylated  $N^{\omega}$ -carboxyalkyl monomers. T\*<sub>1</sub> incorporates monomer **1**, T\*<sub>2</sub> incorporates monomer **2**.

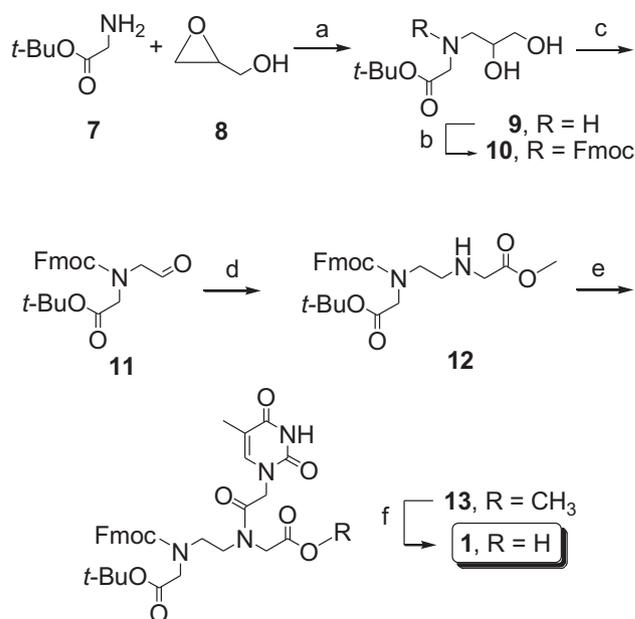
The carboxy termini of the modified mixed purine/pyrimidine decamer PNA sequences were linked to a glycinamide unit. T\*<sub>1</sub> and T\*<sub>2</sub> represent the insertion of the modified **1** and **2**  $N^{\omega}$ -carboxyalkyl monomer units, respectively.

The mixed-base sequence has been chosen since it has been proposed by Nielsen and co-workers<sup>3e</sup> and subsequently used by several groups as a benchmark for the evaluation of the effect of modification of the PNA structure on PNA/DNA thermal stability.<sup>17</sup>

## 2. Results and discussion

### 2.1. Chemistry

The elaboration of monomers **1** and **2** (Fig. 1), suitable for the Fmoc-based oligomerization, took advantage of the chemistry utilized to construct the regular PNA monomers. In particular, the synthesis of the  $N$ -protected monomer **1** started with the  $t$ -Bu–glycine (**7**) glycidol amination,<sup>9</sup> as shown in Scheme 1.  $N$ -Fluorenylmethoxycarbonyl protection of the adduct **9**, and subsequent diol oxidative cleavage, gave the labile aldehyde **11**. Compound **11** was subjected to reductive amination in the presence of methylglycine to obtain the triply protected bis-carboxyalkyl ethylenediamine key intermediate **12**. The 2-(7-aza-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) promoted condensation of **12** with thymine-1-yl-acetic acid gave the expected tertiary amide **13**. Careful LiOH-mediated hydrolysis preserves the base-labile Fmoc group, affording the target monomer unit **1**.



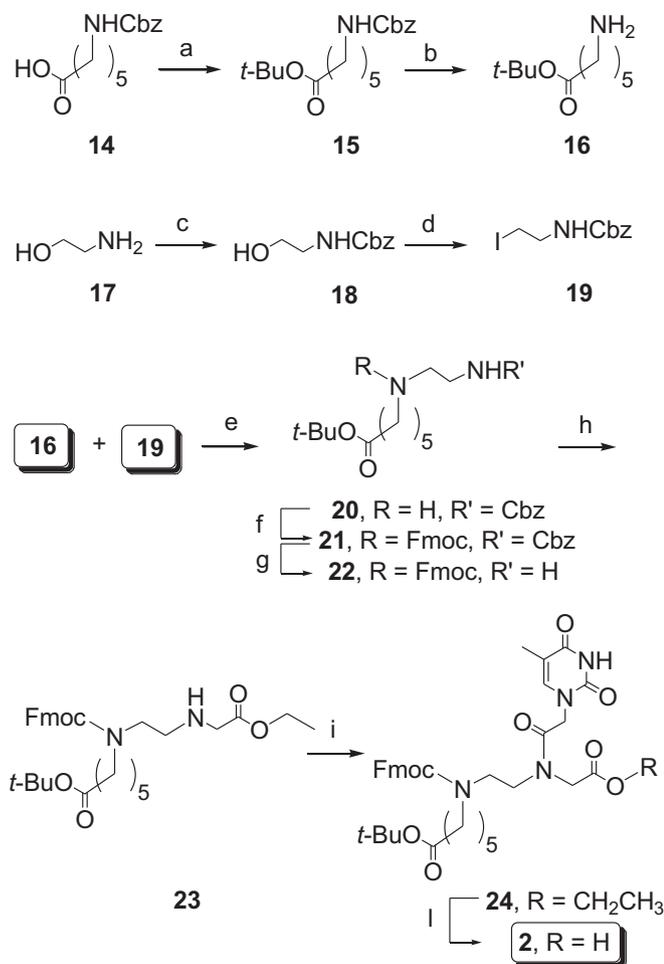
Scheme 1. Synthesis of the PNA monomer **1**. Reagents and conditions: (a) DMF, DIPEA, 70 °C, 3 days, 41%; (b) fluorenylmethoxycarbonyl chloride (Fmoc–Cl), NaHCO<sub>3</sub>, 1,4-dioxane/H<sub>2</sub>O, 18 h, 63%; (c) NaIO<sub>4</sub>, THF/H<sub>2</sub>O, 2 h, 97%; (d) H<sub>2</sub>NCH<sub>2</sub>COOCH<sub>3</sub>, NaH–B(AcO)<sub>3</sub>, triethylamine in CH<sub>2</sub>Cl<sub>2</sub>, 18 h, 70%; (e) thymine-1-yl-acetic acid, Et<sub>3</sub>N, HATU in DMF, 18 h, 49%; (f) LiOH·H<sub>2</sub>O, 1,4-dioxane/H<sub>2</sub>O, 0 °C, 30 min, 69%.

The synthesis of compound **2** required a different strategy, due to the low yields obtained in the glycidol opening induced by the  $tert$ -butyl ester of the 6-aminocaproic acid **16** (see Experimental section). A better electrophile was devised in the benzyl 2-iodoethylcarbamate **19**<sup>18</sup> (Scheme 2). The nucleophilic displacement gave the secondary amine **20**, containing the Cbz-protected ethylenediamine core. Compound **20**, after a straightforward protective group adjustment and a subsequent reductive amination, produced the fully protected bis-carboxyalkyl ethylenediamine key intermediate **23**. Compound **23** was reacted with thymine-1-acetic acid and benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP), as condensing agent, and gave the amide **24**. Finally, after careful chemoselective hydrolysis of the ethyl ester of **24**, the required monomer **2** was obtained in acceptable yields.

The oligomers **3–6** were manually assembled in a stepwise fashion on a Rink amide NOVA-PEG resin solid support. The unmodified PNA monomers were coupled using 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU). HATU was used for the coupling reactions involving the less reactive secondary amino groups of the modified monomers **1** and **2**. The decamers were detached from the solid support and quantitatively deprotected from the  $tert$ -butyl protecting groups, using a 9:1 mixture of trifluoroacetic acid and  $m$ -cresol. The water-soluble oligomers were purified by RP-HPLC, yielding the desired **3–6** as pure compounds. Their identity was confirmed by MALDI-TOF mass spectrometry.

### 2.2. Hybridization studies

In order to verify the ability of decamers **3–6** to bind to complementary DNA, UV-monitored melting experiments were performed mixing the water-soluble oligomers with the complementary antiparallel deoxyribonucleic strands (5 μM concentration each strand, λ=260 nm). Table 1 presents the thermal stability studies of the duplexes formed between the modified PNAs and the DNA antiparallel strand, in comparison with the unmodified PNA.



**Scheme 2.** Synthesis of the PNA monomer **2**. Reagents and conditions: (a) *tert*-butanol, DMAP, DCC, CH<sub>2</sub>Cl<sub>2</sub>, 18 h, 58%; (b) H<sub>2</sub>, Pd/C (10% w/w), acetic acid, methanol, 1 h and 30 min, quant.; (c) Cbz–Cl, CH<sub>2</sub>Cl<sub>2</sub>, 0 °C (2 h) → rt, 18 h, quant.; (d) I<sub>2</sub>, imidazole, PPh<sub>3</sub>, CH<sub>2</sub>Cl<sub>2</sub>, 3 h, 77%; (e) K<sub>2</sub>CO<sub>3</sub>, CH<sub>3</sub>CN, reflux, 18 h, 67%; (f) fluorenylmethoxycarbonyl chloride (Fmoc–Cl), NaHCO<sub>3</sub>, 1,4-dioxane/H<sub>2</sub>O, 18 h, 97%; (g) H<sub>2</sub>, Pd/C (10% w/w), acetic acid, methanol, 1 h, quant.; (h) ethyl glyoxalate, NaHB(AcO)<sub>3</sub>, triethylamine in CH<sub>2</sub>Cl<sub>2</sub>, 18 h, 25%; (i) thymine-1-yl-acetic acid, Et<sub>3</sub>N, PyBOP in DMF, 18 h, 70%; (l) LiOH·H<sub>2</sub>O, 1,4-dioxane/H<sub>2</sub>O, 30 min, 30%.

**Table 1**  
Thermal stabilities (*T<sub>m</sub>*, °C) of modified PNA/DNA duplexes

Entry	PNA	Antiparallel DNA duplex <sup>a</sup>	DNA mis-matched <sup>b</sup>
1	Ac–GTAGATCACT–Gly–NH <sub>2</sub> (PNA sequence) <sup>8a</sup>	48.6	36.4
2	GTAGAT <sub>1</sub> CACT–Gly–NH <sub>2</sub> ( <b>3</b> )	43.2	33.5
3	GT <sub>1</sub> AGAT <sub>1</sub> CACT <sub>1</sub> –Gly–NH <sub>2</sub> ( <b>4</b> )	40.7	34.4
4	GTAGAT <sub>2</sub> CACT–Gly–NH <sub>2</sub> ( <b>5</b> )	44.8	30.8
5	GT <sub>2</sub> AGAT <sub>2</sub> CACT <sub>2</sub> –Gly–NH <sub>2</sub> ( <b>6</b> )	44.1	35.6
6	5′-GTAGATCACT-3′ (DNA sequence) <sup>9</sup>	33.5	26.5

<sup>a</sup> 5′-AGTGATCTAC-3′.

<sup>b</sup> 5′-AGTGGTCTAC-3′.

The data obtained clearly demonstrates that the distance of the negative charged carboxy group from the oligoamide backbone strongly affects the PNA/DNA duplex stability. In particular, when the  $\gamma$ -nitrogen brings an acetic acid substituent (with a single methylene connecting the oligoamide backbone and the charged group, entry 2), a drop of 5.4 °C in *T<sub>m</sub>* of the carboxypeptoid-PNA/DNA (ap) duplex is observed, when compared with unmodified PNA (entry 1). Triple insertion of monomer **1** (entry 3), results in a decrease of 2.6 °C per *N*-acetyl unit, showing no *N*<sup>γ</sup>-substitution detrimental additive effects on the annealing properties. In both

cases the ability to discriminate closely related sequences is magnified, with respect to the unmodified PNA.

For the binding of the *N*<sup>γ</sup>-caproic acid derivatives with the full-matched antiparallel DNA, the table shows an evident increase of the affinity (entries 4 and 5), when compared with the modified sequences with shorter side chains (entries 2 and 3). Comparison with the corresponding aegPNA shows, for the single insertion, a 3.8 °C *T<sub>m</sub>* drop, while, for triple substitution, a *T<sub>m</sub>* decrease of 1.5 °C per *N*<sup>γ</sup>-alkylated monomer. It is also worth noting, in both **5** and **6**, the slight increase of the binding specificity ( $\Delta T_m=5.6$  °C and 0.8 °C, entries 4 and 5) respect to unmodified PNA.

In previous studies, reporting the performances of backbone modified PNA containing negatively charged monomers derived from amino acids, the drop in melting temperature was found to be 3.3 °C in the case of the L-Asp monomer and 2.3 °C in the case of D-Glu.<sup>3e</sup> The present results are in line with these data, with a decrease in melting temperatures, which still allows stronger binding than natural DNA (entry 6). Thus it is possible to introduce negatively charged groups via alkylation of the amide nitrogen in the PNA backbone without significant loss of stability of the PNA/DNA duplex, provided that a five methylene spacer is used.

### 3. Conclusions

In this contribution, we have constructed two orthogonally protected *N*<sup>γ</sup>- $\omega$ -carboxy alkylated units. The successful insertion in PNA-based decamers, through standard solid-phase synthesis protocols, and the following hybridization studies, in the presence of DNA antiparallel strand, demonstrate that the N-substitution with negative charged groups is compatible with the formation of a stable PNA/DNA duplex. The present study also extends the observation that correlates the efficacy of the nucleic acids hybridization with the length of the *N*<sup>γ</sup> alkyl substitution,<sup>8a</sup> expanding the validity also to *N*<sup>γ</sup>- $\omega$ -negative charged side chains.

The newly produced structures can create new possibilities for PNA with functional groups enabling further improvement in their ability to perform gene-regulation.

### 4. Experimental section

#### 4.1. General methods

All reactions involving air or moisture sensitive reagents were carried out under a dry argon or nitrogen atmosphere using freshly distilled solvents. Tetrahydrofuran (THF) was distilled from LiAlH<sub>4</sub> under argon. Toluene and CH<sub>2</sub>Cl<sub>2</sub> were distilled from CaH<sub>2</sub>. Glassware was flame-dried (0.05 Torr) prior to use. When necessary, compounds were dried in vacuo over P<sub>2</sub>O<sub>5</sub> or by azeotropic removal of water with toluene under reduced pressure. Starting materials and reagents purchased from commercial suppliers were generally used without purification unless otherwise mentioned. Reaction temperatures were measured externally; reactions were monitored by TLC on Merck silica gel plates (0.25 mm) and visualized by UV light, I<sub>2</sub>, or by spraying with H<sub>2</sub>SO<sub>4</sub>/Ce(SO<sub>4</sub>)<sub>2</sub>, phosphomolybdic acid or ninhydrin solutions and drying. Flash chromatography was performed on Merck silica gel 60 (particle size: 0.040–0.063 mm) and the solvents employed were of analytical grade. Yields refer to chromatographically and spectroscopically (<sup>1</sup>H and <sup>13</sup>C NMR) pure materials. The NMR spectra were recorded on Bruker DRX 400 (<sup>1</sup>H at 400.13 MHz, <sup>13</sup>C at 100.03 MHz), Bruker DRX 250 (<sup>1</sup>H at 250.13 MHz, <sup>13</sup>C at 62.89 MHz), and Bruker DRX 300 (<sup>1</sup>H at 300.10 MHz, <sup>13</sup>C at 75.50 MHz) spectrometers. Chemical shifts ( $\delta$ ) are reported in parts per million relatively to the residual solvent peak (CHCl<sub>3</sub>,  $\delta=7.26$ , <sup>13</sup>CDCl<sub>3</sub>,  $\delta=77.0$ ; CD<sub>2</sub>HOD,  $\delta=3.34$ , <sup>13</sup>CD<sub>3</sub>OD,  $\delta=49.0$ ) and the multiplicity of each signal is designated by the following abbreviations: s, singlet; d, doublet; t, triplet; q, quartet;

quint, quintuplet; m, multiplet; br, broad. Coupling constants ( $J$ ) are quoted in hertz. Homonuclear decoupling, COSY-45, and DEPT experiments completed the full assignment of each signal. Elemental analyses were performed on a CHNS-O FlashEA apparatus (Thermo Electron Corporation) and are reported in percent abundance. High resolution ESI-MS spectra were performed on a Q-Star Applied Biosystem mass spectrometer. ESI-MS analysis in positive ion mode was performed using a Finnigan LCQ Deca ion trap mass spectrometer (ThermoFinnigan, San José, CA, USA) and the mass spectra were acquired and processed using the Xcalibur software provided by Thermo Finnigan. Samples were dissolved in 1:1 CH<sub>3</sub>OH/H<sub>2</sub>O, 0.1% formic acid, and infused in the ESI source by using a syringe pump; the flow rate was 5  $\mu$ L/min. The capillary voltage was set at 4.0 V, the spray voltage at 5 kV, and the tube lens offset at  $-40$  V. The capillary temperature was 220 °C. MALDI-TOF mass spectrometric analyses were performed on a PerSeptive Biosystems Voyager-De Pro MALDI mass spectrometer in the linear mode using  $\alpha$ -cyano-4-hydroxycinnamic acid as the matrix. HPLC analyses were performed on a Jasco BS 997-01 series, equipped with a quaternary pumps Jasco PU-2089 Plus, and an UV detector Jasco MD-2010 Plus. The resulting residues were purified by semi-preparative reverse-phase C18 (Waters, Bondapak, 10  $\mu$ m, 125 Å, 7.8  $\times$  300 mm).

## 4.2. Chemistry

**4.2.1. tert-Butyl 2-(2,3-dihydroxypropylamino)acetate (9).** To a solution of glycidol (**8**, 436  $\mu$ L, 6.56 mmol) in DMF (5 mL), glycine tert-butyl ester (**7**, 1.00 g, 5.96 mmol) in DMF (10 mL), and DIPEA (1600  $\mu$ L, 8.94 mmol) were added. The reaction mixture was refluxed for 3 days. NaHCO<sub>3</sub> (0.50 g, 5.96 mmol) was added and the solvent was concentrated in vacuo to give the crude product, which was purified by flash chromatography (CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>OH/NH<sub>3</sub> 2.0 M solution in ethyl alcohol from 100:0:0.1 to 88:12:0.1) to give **9** (0.50 g, 41%) as a pale yellow oil. Found: C, 52.7; H, 9.4. C<sub>9</sub>H<sub>19</sub>NO<sub>4</sub> requires C, 52.67; H, 9.33%;  $R_f$  (97:3:0.1 CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>OH/NH<sub>3</sub> 2.0 M solution in ethyl alcohol) 0.36;  $\delta_H$  (400.13 MHz, CDCl<sub>3</sub>) 1.42 (9H, s, (CH<sub>3</sub>)<sub>3</sub>C), 2.62 (1H, dd,  $J$  12.0, 7.7 Hz, CHHCH(OH)CH<sub>2</sub>OH), 2.71 (1H, dd,  $J$  12.0, 2.9 Hz, CHHCH(OH)CH<sub>2</sub>OH), 3.28 (2H, br s, CH<sub>2</sub>COO-*t*-Bu), 3.51 (1H, dd,  $J$  11.0, 5.4 Hz, CH<sub>2</sub>CH(OH)CHHOH), 3.62 (1H, dd,  $J$  11.0, 1.2 Hz, CH<sub>2</sub>CH(OH)CHHOH), 3.72 (1H, m, CH<sub>2</sub>CH(OH)CH<sub>2</sub>OH);  $\delta_C$  (100.03 MHz, CDCl<sub>3</sub>) 29.2, 52.6, 53.1, 66.4, 71.6, 82.7, 172.8;  $m/z$  (ES) 206 (MH<sup>+</sup>); (HRES) MH<sup>+</sup>, found 206.1390. C<sub>9</sub>H<sub>20</sub>NO<sub>4</sub> requires 206.1387.

**4.2.2. (9H-Fluoren-9-yl)methyl(tert-butoxycarbonyl)methyl 2,3-dihydroxypropylcarbamate (10).** To a solution of **9** (0.681 g, 3.33 mmol) in a 1:1 mixture of 1,4-dioxane/water (46 mL), NaHCO<sub>3</sub> (0.559 g, 6.66 mmol) was added. The mixture was sonicated until complete dissolution and Fmoc-Cl (1.03 g, 3.99 mmol) was added. The reaction mixture was stirred for 18 h, then, through addition of a saturated solution of NaHSO<sub>4</sub>, the pH was adjusted to 3 and the solvent was concentrated in vacuo to remove the excess of 1,4-dioxane. The water layer was extracted with CH<sub>2</sub>Cl<sub>2</sub> (3  $\times$  50 mL), the organic phase was dried over MgSO<sub>4</sub>, filtered, and the solvent evaporated in vacuo to give the crude product, which was purified by flash chromatography (CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>OH from 100:0 to 90:10) to give **10** (0.90 g, 63%) as a pale yellow oil. Found: C, 67.4; H, 6.9. C<sub>24</sub>H<sub>29</sub>NO<sub>6</sub> requires C, 67.43; H, 6.84%;  $R_f$  (95:5:0.1 CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>OH/NH<sub>3</sub> 2.0 M solution in ethyl alcohol) 0.44;  $\delta_H$  (300.10 MHz, CDCl<sub>3</sub>, mixture of rotamers) 1.45 (9H, s, (CH<sub>3</sub>)<sub>3</sub>C), 3.04–3.25 (1.7H, m, CH<sub>2</sub>CH(OH)CH<sub>2</sub>OH), 3.40 (0.3H, m, CH<sub>2</sub>CH(OH)CH<sub>2</sub>OH), 3.43–3.92 (3H, m, CH<sub>2</sub>CH(OH)CH<sub>2</sub>OH, CH<sub>2</sub>CH(OH)CH<sub>2</sub>OH), 3.93 (2H, br s, CH<sub>2</sub>COO-*t*-Bu), 4.22 (0.9H, m, CH-Fmoc and CH<sub>2</sub>-Fmoc), 4.42 (1.4H, br d,  $J$  9.0 Hz, CH<sub>2</sub>-Fmoc), 4.61 (0.7H, m,  $J$  9.0 Hz, CH-Fmoc), 7.29 (2H, br t,  $J$  7.0 Hz, Ar. (Fmoc)), 7.38 (2H, br t,  $J$  7.0 Hz, Ar. (Fmoc)), 7.57 (2H, br d,  $J$  9.0 Hz, Ar. (Fmoc)), 7.76 (2H, br d,  $J$  9.0 Hz, Ar. (Fmoc));  $\delta_C$  (75.50 MHz, CDCl<sub>3</sub>,

mixture of rotamers) 28.2, 47.4, 52.1, 52.3, 52.9, 53.2, 63.5, 64.0, 67.3, 68.1, 68.5, 70.1, 70.5, 83.1, 120.1, 120.2, 124.9, 125.2, 127.3, 127.9, 128.0, 141.5, 143.8, 156.4, 157.3, 170.4, 171.3;  $m/z$  (ES) 428 (MH<sup>+</sup>); (HRES) MH<sup>+</sup>, found 428.2070. C<sub>24</sub>H<sub>30</sub>NO<sub>6</sub> requires 428.2068.

**4.2.3. (9H-Fluoren-9-yl)methyl(tert-butoxycarbonyl)methyl-formylmethylcarbamate (11).** To a solution of **10** (0.80 g, 1.87 mmol) in a 5:1 mixture of THF and water (5 mL), sodium periodate (0.44 g, 2.06 mmol) was added in one portion. The mixture was sonicated for 15 min and stirred for another 2 h at room temperature. The reaction mixture was filtered, the filtrate was washed with CH<sub>2</sub>Cl<sub>2</sub>, and the solvent evaporated in vacuo. The crude product was dissolved in CH<sub>2</sub>Cl<sub>2</sub>/H<sub>2</sub>O, and the organic phase was dried over MgSO<sub>4</sub>, filtered and the solvent evaporated in vacuo to give the labile aldehyde **11** (0.72 g, 97%), as white solid;  $R_f$  (92:8 CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>OH) 0.56; crude **11** was used immediately in the subsequent reductive amination reaction;  $\delta_H$  (300.10 MHz, CDCl<sub>3</sub>, mixture of rotamers) 1.43 (4.05H, s, (CH<sub>3</sub>)<sub>3</sub>C), 1.45 (4.95H, s, (CH<sub>3</sub>)<sub>3</sub>C), 3.81 (0.9H, br s, CH<sub>2</sub>COO-*t*-Bu), 3.99 (2H, br s, CH<sub>2</sub>CHO and CH<sub>2</sub>COO-*t*-Bu, overlapped), 4.08 (1.1H, s, CH<sub>2</sub>CHO), 4.22–4.19 (1H, m, CH-Fmoc), 4.42 (1.1H, d,  $J$  6.0 Hz, CH<sub>2</sub>-Fmoc), 4.50 (0.9H, d,  $J$  6.0 Hz, CH<sub>2</sub>-Fmoc), 7.29 (0.9H, br t,  $J$  7.0 Hz, Ar. (Fmoc)), 7.38 (1.1H, t,  $J$  7.0 Hz, Ar. (Fmoc)), 7.49 (0.9H, d,  $J$  9.0 Hz, Ar. (Fmoc)), 7.56 (1.1H, d,  $J$  9.0 Hz, Ar. (Fmoc)), 7.73 (2H, m, Ar. (Fmoc)), 9.35 (0.45H, br s, CHO), 9.64 (0.55H, br s, CHO);  $\delta_C$  (75.50 MHz, CDCl<sub>3</sub>) 28.2, 47.3, 50.6, 50.8, 57.8, 58.4, 68.1, 68.6, 82.6, 82.7, 120.2, 124.9, 125.2, 127.3, 128.0, 141.5, 143.8, 143.9, 156.0, 156.4, 168.6, 168.7, 198.7;  $m/z$  (ES) 396 (MH<sup>+</sup>); (HRES) MH<sup>+</sup>, found 396.1809. C<sub>23</sub>H<sub>26</sub>NO<sub>5</sub> requires 396.1805.

**4.2.4. (9H-Fluoren-9-yl)methyl(tert-butoxycarbonyl)methyl 2-((methoxycarbonyl)methylamino)ethylcarbamate (12).** To a solution of crude aldehyde **11** (0.72 g, 1.83 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub> (12 mL), a solution of glycine methyl ester hydrochloride (0.30 g, 2.39 mmol) and Et<sub>3</sub>N (0.41 mL, 2.93 mmol) was added. The reaction mixture was stirred for 1 h. Sodium triacetoxyborohydride (0.78 g, 3.66 mmol) was then added and the reaction mixture was stirred for 18 h at room temperature. The resulting mixture was washed with an aqueous saturated solution of NaHCO<sub>3</sub> and the aqueous phase extracted with CH<sub>2</sub>Cl<sub>2</sub> (3  $\times$  20 mL). The organic phase was dried over MgSO<sub>4</sub>, filtered, and the solvent evaporated in vacuo to give the crude product, which was purified by flash chromatography (AcOEt/petroleum ether/NH<sub>3</sub> 2.0 M solution in ethyl alcohol from 40:60:0.1 to 90:10:0.1) to give **12** (0.60 g, 70%) as a colorless oil. Found: C, 66.7; H, 6.9. C<sub>26</sub>H<sub>32</sub>N<sub>2</sub>O<sub>6</sub> requires C, 66.65; H, 6.88%;  $R_f$  (98:2:0.1 CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>OH/NH<sub>3</sub> 2.0 M solution in ethyl alcohol) 0.63;  $\delta_H$  (400.13 MHz, CDCl<sub>3</sub>, mixture of rotamers) 1.45 (9H, s, (CH<sub>3</sub>)<sub>3</sub>C), 2.56 (0.9H, t,  $J$  6.0 Hz, N(Fmoc)CH<sub>2</sub>CH<sub>2</sub>NH), 2.83 (1.1H, t,  $J$  6.0 Hz, N(Fmoc)CH<sub>2</sub>CH<sub>2</sub>NH), 3.27 (0.9H, t,  $J$  6.0 Hz, N(Fmoc)CH<sub>2</sub>CH<sub>2</sub>NH), 3.29 (0.9H, s, CH<sub>2</sub>COOMe), 3.44 (1.1H, s, CH<sub>2</sub>COOMe), 3.49 (1.1H, t,  $J$  6.0 Hz, N(Fmoc)CH<sub>2</sub>CH<sub>2</sub>NH), 3.72 (3H, s, CH<sub>3</sub>), 3.91 (0.9H, s, CH<sub>2</sub>COO-*t*-Bu), 3.96 (1.1H, s, CH<sub>2</sub>COO-*t*-Bu), 4.21 (0.45H, t,  $J$  6.0 Hz, CH<sub>2</sub>CHFmoc), 4.26 (0.55H, t,  $J$  6.0 Hz, CH<sub>2</sub>CHFmoc), 4.37 (1.1H, d,  $J$  6.0 Hz, CH<sub>2</sub>CHFmoc), 4.51 (0.9H, d,  $J$  6.0 Hz, CH<sub>2</sub>CHFmoc), 7.29 (2H, t,  $J$  7.0 Hz, Ar. (Fmoc)), 7.39 (2H, t,  $J$  7.0 Hz, Ar. (Fmoc)), 7.58 (2H, m, Ar. (Fmoc)), 7.75 (2H, d,  $J$  7.0 Hz, Ar. (Fmoc));  $\delta_C$  (100.03 MHz, CDCl<sub>3</sub>) 27.8, 47.0, 47.2, 47.4, 48.2, 48.6, 50.1, 50.3, 50.5, 53.3, 67.2, 67.6, 81.5, 81.7, 119.7, 124.7, 124.9, 126.8, 127.5, 141.0, 143.7, 156.0, 156.2, 168.7, 169.4, 171.9, 172.1;  $m/z$  (ES) 469 (MH<sup>+</sup>); (HRES) MH<sup>+</sup>, found 469.2341. C<sub>26</sub>H<sub>33</sub>N<sub>2</sub>O<sub>6</sub> requires 469.2333.

**4.2.5. Compound 13.** To a solution of **12** (0.60 g, 1.28 mmol) in DMF (30 mL), thymine-1-acetic acid (0.35 g, 1.90 mmol), HATU (0.73 g, 1.90 mmol), and triethylamine (0.54 mL, 3.84 mmol) were added. The reaction mixture was stirred for 18 h, concentrated in vacuo, dissolved in CH<sub>2</sub>Cl<sub>2</sub> (20 mL), and washed with 1 M HCl solution. The aqueous layer was extracted with CH<sub>2</sub>Cl<sub>2</sub> (3  $\times$  20 mL). The combined

organic phases were dried over  $\text{MgSO}_4$ , filtered, and the solvent evaporated in vacuo to give a crude material, which was purified by flash chromatography (AcOEt/petroleum ether from 30:70 to 100:0) to give **13** (0.40 g, 49%) as yellow oil. Found: C, 62.5; H, 6.1.  $\text{C}_{33}\text{H}_{39}\text{N}_4\text{O}_9$  requires C, 62.45; H, 6.03%;  $R_f$  (8:2 AcOEt/petroleum ether) 0.38;  $\delta_{\text{H}}$  (300.10 MHz,  $\text{CDCl}_3$ , mixture of rotamers) 1.39–1.44 (9H, m,  $(\text{CH}_3)_3\text{C}$ ), 1.88 (3H, br s,  $\text{CH}_3$ -thymine), 2.99 (0.3H, m,  $\text{CH}_2\text{CH}_2\text{N}(\text{Fmoc})$ ), 3.08 (0.3H, m,  $\text{CH}_2\text{CH}_2\text{N}(\text{Fmoc})$ ), 3.52 (2.8H, m,  $\text{CH}_2\text{CH}_2\text{N}(\text{Fmoc})$  and  $\text{CH}_2\text{CH}_2\text{N}(\text{Fmoc})$ ), 3.77–3.89 (3.6H, m,  $\text{CH}_2\text{CH}_2\text{N}(\text{Fmoc})$  and  $\text{CH}_3\text{OOC}$ ), 3.96–4.38 (6H, m,  $\text{CH}_2$ -thymine,  $\text{CH}_2\text{COOCH}_3$ ,  $\text{CH}_2\text{COO}-t\text{-Bu}$ ), 4.45–4.80 (3H, m,  $\text{CH}(\text{Fmoc})$  and  $\text{CH}_2\text{CH}(\text{Fmoc})$ ), 6.90–7.06 (1H, complex signal,  $\text{CH}$ -thymine), 7.28 (2H, m, Ar. (Fmoc)), 7.41 (2H, t,  $J$  7.0 Hz, Ar (Fmoc)), 7.55 (2H, d,  $J$  7.0 Hz, Ar. (Fmoc)), 7.75 (2H, t,  $J$  7.0 Hz, Ar. (Fmoc)), 8.86 (1H, br s,  $\text{NH}$ -thymine);  $\delta_{\text{C}}$  (75.5 MHz,  $\text{CDCl}_3$ ) 12.5, 28.2, 31.6, 36.6, 47.2, 47.4, 47.5, 47.8, 48.2, 49.1, 50.6, 51.8, 52.1, 52.5, 53.0, 66.5, 68.2, 82.3, 82.5, 110.5, 120.2, 124.5, 124.8, 125.14, 125.3, 127.3, 127.4, 127.5, 128.0, 128.1, 141.3, 141.4, 143.8, 144.0, 151.1, 156.4, 162.7, 164.4, 169.1, 169.2;  $m/z$  (ES) 635 ( $\text{MH}^+$ ); (HRES)  $\text{MH}^+$ , found 635.2741.  $\text{C}_{33}\text{H}_{39}\text{N}_4\text{O}_9^+$  requires 635.2712.

**4.2.6. Compound 1.** To a solution of **13** (0.40 g, 0.63 mmol) in a 1:1 mixture of 1,4-dioxane/water (8 mL) at 0 °C, LiOH monohydrate (58 mg, 1.39 mmol) was added. The reaction mixture was stirred for 30 min and a saturated solution of  $\text{NaHSO}_4$  was added until pH  $\sim$  3. The aqueous layer was extracted with  $\text{CH}_2\text{Cl}_2$  ( $3 \times 15$  mL) and once with AcOEt. The combined organic phases were dried over  $\text{MgSO}_4$ , filtered, and the solvent evaporated in vacuo to give a crude material, which was purified by flash chromatography ( $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}/\text{AcOH}$  from 95:5:0.1 to 80:20:0.1) to give **1** (0.27 g, 69%) as a white solid. Found: C, 62.0; H, 6.0.  $\text{C}_{32}\text{H}_{36}\text{N}_4\text{O}_9$  requires C, 61.93; H, 5.85%;  $R_f$  (9:1:0.1  $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}/\text{NH}_3$  2.0 M solution in ethyl alcohol) 0.12;  $\delta_{\text{H}}$  (250.13 MHz,  $\text{CDCl}_3$ , mixture of rotamers) 1.39–1.43 (9H, m,  $(\text{CH}_3)_3\text{C}$ ), 1.83 (3H, br s,  $\text{CH}_3$  (thymine)), 3.03 (0.3H, m,  $\text{CH}_2\text{CH}_2\text{N}(\text{Fmoc})$ ), 3.17 (0.3H, m,  $\text{CH}_2\text{CH}_2\text{N}(\text{Fmoc})$ ), 3.55–3.72 (2.8H, m,  $\text{CH}_2\text{CH}_2\text{N}(\text{Fmoc})$  and  $\text{CH}_2\text{CH}_2\text{N}(\text{Fmoc})$ ), 3.95–4.06 (6.6H m,  $\text{CH}_2\text{CH}_2\text{N}(\text{Fmoc})$ ,  $\text{CH}_2$ -thymine,  $\text{CH}_2\text{COOH}$ ,  $\text{CH}_2\text{COO}-t\text{-Bu}$ ), 4.14–4.77 (3H, m,  $\text{CH}(\text{Fmoc})$  and  $\text{CH}_2\text{CH}(\text{Fmoc})$ ), 6.97–7.11 (1H, complex signal,  $\text{CH}$ -thymine), 7.23–7.40 (4H, m, Ar. (Fmoc)), 7.55 (2H, d,  $J$  7.0 Hz, Ar. (Fmoc)), 7.75 (2H, m, Ar. (Fmoc)), 10.00 (1H, br s,  $\text{NH}$ -thymine);  $\delta_{\text{C}}$  (75.50 MHz,  $\text{CDCl}_3$ ) 12.3, 28.2, 29.9, 46.4, 47.3, 48.7, 50.2, 50.9, 53.6, 68.3, 82.3, 82.6, 110.8, 120.2, 124.9, 125.2, 125.3, 127.3, 127.5, 128.0, 141.4, 142.1, 143.8, 144.0, 151.7, 156.6, 165.0, 165.2, 168.2, 169.0, 169.2, 172.3;  $m/z$  (ES) 621 ( $\text{MH}^+$ ); (HRES)  $\text{MH}^+$ , found 621.2541.  $\text{C}_{32}\text{H}_{37}\text{N}_4\text{O}_9^+$  requires 621.2555.

**4.2.7. Benzyl 5-(tert-butoxycarbonyl)pentylcarbamate (15).** To a solution of **14** (3.00 g, 11.3 mmol), DMAP (0.14 g, 1.13 mmol), and  $t\text{-BuOH}$  (1.30 mL, 13.9 mmol) in dry  $\text{CH}_2\text{Cl}_2$  (5.0 mL), a solution of DCC (13.6 mL, 13.6 mmol, 1.0 M in  $\text{CH}_2\text{Cl}_2$ ) was added. The reaction mixture was filtered, the filtrate washed with  $\text{CH}_2\text{Cl}_2$ , and the solvent evaporated in vacuo to give the crude product, which was purified by flash chromatography (AcOEt/petroleum ether from 10:90 to 100:0) to give **15** (2.10 g, 58%) as white solid. Found: C, 67.2; H, 8.4.  $\text{C}_{18}\text{H}_{27}\text{NO}_4$  requires C, 67.26; H, 8.47%;  $R_f$  (97:3  $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$ ) 0.84;  $\delta_{\text{H}}$  (400.13 MHz,  $\text{CDCl}_3$ ) 1.31 (2H, q,  $J$  6.5 Hz,  $\text{CH}_2\text{CH}_2\text{CH}_2\text{COO}-t\text{-Bu}$ ), 1.41 (9H, s,  $\text{COOC}(\text{CH}_3)_3$ ), 1.48 (2H, q,  $J$  6.5 Hz,  $\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}$ ), 1.56 (2H, q,  $J$  6.5 Hz,  $\text{CH}_2\text{CH}_2\text{CH}_2\text{COO}-t\text{-Bu}$ ), 2.18 (2H, t,  $J$  6.5 Hz,  $\text{CH}_2\text{CH}_2\text{CH}_2\text{COO}-t\text{-Bu}$ ), 3.15 (2H, q,  $J$  6.5 Hz,  $\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}$ ), 4.91 (1H, br s,  $\text{NH}$ ), 5.07 (2H, br s,  $\text{CH}_2\text{Bn}$ ), 7.32 (5H, m, Ar.);  $\delta_{\text{C}}$  (75.50 MHz,  $\text{CDCl}_3$ ) 24.5, 26.0, 28.0, 29.5, 35.3, 40.8, 66.4, 79.9, 127.9, 128.0, 128.3, 136.6, 156.3, 172.8;  $m/z$  (ES) 322 ( $\text{MH}^+$ ); (HRES)  $\text{MH}^+$ , found 322.2009.  $\text{C}_{18}\text{H}_{28}\text{NO}_4^+$  requires 322.2013.

**4.2.8. tert-Butyl 6-aminohexanoate (16).** To a solution of **15** (1.95 g, 6.07 mmol) in dry MeOH (150 mL), acetic acid (1.39 mL, 24.0 mmol)

and palladium on charcoal (10% w/w, 0.19 g) were added. The reaction mixture was stirred under a hydrogen atmosphere at room temperature for 1 h and filtered through Celite. The solvent was evaporated in vacuo to give crude **16** (1.13 g, 100%, colorless oil), which was used in the next step without purification;  $R_f$  (95:5  $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$ ) 0.46;  $\delta_{\text{H}}$  (400.13 MHz,  $\text{CDCl}_3$ ) 1.33 (2H, q,  $J$  6.5 Hz,  $\text{CH}_2\text{CH}_2\text{CH}_2\text{COO}-t\text{-Bu}$ ), 1.39 (9H, s,  $\text{COOC}(\text{CH}_3)_3$ ), 1.55 (2H, q,  $J$  6.5 Hz,  $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}$ ), 1.62 (2H, q,  $J$  6.5 Hz,  $\text{CH}_2\text{CH}_2\text{CH}_2\text{COO}-t\text{-Bu}$ ), 2.17 (2H, t,  $J$  6.5 Hz,  $\text{CH}_2\text{CH}_2\text{CH}_2\text{COO}-t\text{-Bu}$ ), 2.84 (2H, t,  $J$  6.5 Hz,  $\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}$ );  $\delta_{\text{C}}$  (75.50 MHz,  $\text{CDCl}_3$ ) 24.3, 25.8, 27.2, 28.0, 35.1, 39.4, 80.2, 177.2;  $m/z$  (ES) 188 ( $\text{MH}^+$ ); (HRES)  $\text{MH}^+$ , found 188.1653.  $\text{C}_{10}\text{H}_{22}\text{NO}_2^+$  requires 188.1645.

**4.2.9. Benzyl 2-hydroxyethylcarbamate (18).** To a solution of ethanolamine (**17**, 2.00 g, 32.8 mmol) in dry  $\text{CH}_2\text{Cl}_2$  (30 mL) at 0 °C, a solution  $\text{Cbz}-\text{Cl}$  (3.73 mL, 26.2 mmol) in dry  $\text{CH}_2\text{Cl}_2$  (20 mL) was slowly added. The reaction mixture was stirred for 2 h at 0 °C and at room temperature for 18 h. The resulting mixture was washed with an aqueous saturated solution of  $\text{NaHCO}_3$  and the aqueous phase extracted with  $\text{CH}_2\text{Cl}_2$  ( $3 \times 50$  mL). The organic phase was dried over  $\text{MgSO}_4$ , filtered, and the solvent evaporated in vacuo to give crude **18** (5.11 g, 100%, pale yellow oil), which was used in the next step without purification;  $R_f$  (92:8  $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$ ) 0.47;  $\delta_{\text{H}}$  (250.13 MHz,  $\text{CDCl}_3$ ) 3.36 (2H, br t,  $J$  6.5 Hz,  $\text{CH}_2\text{OH}$ ), 3.71 (2H, q,  $J$  6.5 Hz,  $\text{CH}_2\text{NH}$ ), 5.11 (2H, s,  $\text{CH}_2\text{Bn}$ ), 7.35 (5H, m, Ar.);  $\delta_{\text{C}}$  (62.89 MHz,  $\text{CDCl}_3$ ) 43.3, 61.7, 66.7, 127.9, 128.0, 128.4, 136.2, 157.0;  $m/z$  (ES) 196 ( $\text{MH}^+$ ); (HRES)  $\text{MH}^+$ , found 196.0977.  $\text{C}_{10}\text{H}_{14}\text{NO}_2^+$  requires 196.0968.

**4.2.10. Benzyl 2-iodoethylcarbamate (19).** To a solution of  $\text{PPh}_3$  (2.66 g, 10.2 mmol) in  $\text{CH}_2\text{Cl}_2$  (10 mL),  $\text{I}_2$  (2.59 g, 10.2 mmol) in  $\text{CH}_2\text{Cl}_2$  (10 mL) was slowly added. The reaction mixture was stirred for 30 min. Imidazole (1.39 g, 20.4 mmol) in  $\text{CH}_2\text{Cl}_2$  (10 mL) was then added and the reaction mixture was stirred for further 30 min. Finally, **18** (1.00 g, 5.13 mmol) was added and the reaction mixture stirred for 3 h. The resulting mixture was washed with an aqueous saturated solution of  $\text{NaHCO}_3$  and 10% w/w of  $\text{Na}_2\text{S}_2\text{O}_3$  and the aqueous phase extracted with  $\text{CH}_2\text{Cl}_2$  ( $3 \times 25$  mL). The organic phase was dried over  $\text{MgSO}_4$ , filtered, and the solvent evaporated in vacuo to give a crude material, which was purified by flash chromatography (AcOEt/petroleum ether from 0:100 to 100:0) to give **19** (1.20 g, 77%) as white amorphous solid. Found: C, 39.4; H, 4.0.  $\text{C}_{10}\text{H}_{12}\text{INO}_2$  requires C, 39.36; H, 3.96%;  $R_f$  (6:4 AcOEt/petroleum ether) 0.88;  $\delta_{\text{H}}$  (250.13 MHz,  $\text{CDCl}_3$ ) 3.25 (2H, t,  $J$  6.5 Hz,  $\text{CH}_2\text{I}$ ), 3.55 (2H, q,  $J$  6.5 Hz,  $\text{CH}_2\text{NH}$ ), 5.11 (2H, s,  $\text{CH}_2\text{Bn}$ ), 7.36 (5H, m, Ar.);  $\delta_{\text{C}}$  (62.89 MHz,  $\text{CDCl}_3$ ) 5.1, 43.0, 66.5, 127.8, 128.1, 128.2, 136.0, 155.8;  $m/z$  (ES) 306 ( $\text{MH}^+$ ); (HRES)  $\text{MH}^+$ , found 305.9991.  $\text{C}_{10}\text{H}_{13}\text{INO}_2^+$  requires 305.9985.

**4.2.11. Benzyl 2-(5-(tert-butoxycarbonyl)pentylamino)ethylcarbamate (20).** To a solution of **16** (0.35 g, 1.87 mmol) in dry acetonitrile (10 mL), at reflux,  $\text{K}_2\text{CO}_3$  (0.88 g, 6.38 mmol) was added. The reaction mixture was stirred for 10 min. After that, a solution of **19** (0.40 g, 1.31 mmol) in dry acetonitrile (5 mL) was added and the reaction mixture was stirred at reflux 18 h. The product was filtered and the crude was purified by flash column chromatography ( $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$  from 100:0 to 90:10) to give **20** (0.32 g, 67%) as yellow light oil. Found: C, 65.9; H, 8.6.  $\text{C}_{20}\text{H}_{32}\text{N}_2\text{O}_4$  requires C, 65.91; H, 8.85%;  $R_f$  (93:7  $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$ ) 0.71;  $\delta_{\text{H}}$  (300.10 MHz,  $\text{CDCl}_3$ ) 1.35 (2H, q,  $J$  6.5 Hz,  $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}$ ), 1.43 (9H, s,  $\text{COOC}(\text{CH}_3)_3$ ), 1.57 (2H, q,  $J$  6.0 Hz,  $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}$ ), 1.67 (2H, q,  $J$  6.0 Hz,  $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}$ ), 2.21 (2H, t,  $J$  6.0 Hz,  $\text{OOCCH}_2\text{CH}_2$ ), 2.82 (2H, t,  $J$  6.0 Hz,  $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}$ ), 2.99 (2H, t,  $J$  6.0 Hz,  $\text{CONHCH}_2\text{CH}_2\text{NH}$ ), 3.46 (2H, q,  $J$  6.0 Hz,  $\text{CONHCH}_2\text{CH}_2\text{NH}$ ), 5.09 (2H, s,  $\text{CH}_2\text{Ar}$ ), 5.73 (1H, br s,  $\text{NHCOO}$ ), 7.34 (5H, m, Ar.);  $\delta_{\text{C}}$  (75.50 MHz,  $\text{CDCl}_3$ ) 24.4, 26.2, 27.9, 35.0, 39.3, 48.4, 48.6, 66.6, 79.9, 127.9, 128.0, 128.3, 136.1, 156.6, 172.8;  $m/z$

(ES) 365 (MH<sup>+</sup>); (HRES) MH<sup>+</sup>, found 365.2426. C<sub>20</sub>H<sub>33</sub>N<sub>2</sub>O<sub>4</sub><sup>+</sup> requires 365.2435.

**4.2.12. Compound (21).** To a solution of **20** (0.32 g, 0.88 mmol) in a 1:1 mixture of 1,4-dioxane/water (20 mL), NaHCO<sub>3</sub> (148 mg, 1.76 mmol) was added. The mixture was sonicated until complete dissolution and Fmoc-Cl (0.29 g, 1.12 mmol) was added. The reaction mixture was stirred 18 h, then, through addition of a saturated solution of NaHSO<sub>4</sub>, the pH was adjusted to 3 and the solvent was concentrated in vacuo to remove the excess of dioxane. The water layer was extracted with CH<sub>2</sub>Cl<sub>2</sub> (3 × 25 mL), the organic phase was dried over MgSO<sub>4</sub>, filtered, and the solvent evaporated in vacuo to give the crude product, which was purified by flash chromatography (CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>OH from 100:0 to 98:2) to give **21** (0.50 g, 97%) as a yellow light oil. Found: C, 71.7; H, 7.3. C<sub>35</sub>H<sub>42</sub>N<sub>2</sub>O<sub>6</sub> requires C, 71.65; H, 7.22%; R<sub>f</sub> (95:5 CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>OH) 0.61; δ<sub>H</sub> (400.13 MHz, CDCl<sub>3</sub>, mixture of rotamers) 1.08–1.60 (15H, m, CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>N, COOC(CH<sub>3</sub>)<sub>3</sub>), 2.16 (2H, t, J 6.0 Hz, CH<sub>2</sub>COO), 2.85 (0.8H, br s, CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>N), 2.96 (2.4H, CONHCH<sub>2</sub>CH<sub>2</sub>N and CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>N), 3.13 (0.8H, br s, CONHCH<sub>2</sub>CH<sub>2</sub>N), 3.30 (2H, br s, CONHCH<sub>2</sub>CH<sub>2</sub>N), 4.19 (1H, br s, CH<sub>2</sub>CHFmoc), 4.53–4.57 (2H, br s, CH<sub>2</sub>CHFmoc), 5.05 (2H, br s, CH<sub>2</sub>Ar), 7.29 (7H, m, Ar. (Cbz) and Ar. (Fmoc)), 7.38 (2H, t, J 7.0 Hz, Ar. (Fmoc)), 7.55 (2H, d, J 7.0 Hz, Ar. (Fmoc)), 7.76 (2H, t, J 7.0 Hz, Ar. (Fmoc)); δ<sub>C</sub> (62.89 MHz, CDCl<sub>3</sub>) 24.5, 25.9, 27.8, 27.9, 35.2, 39.2, 39.6, 46.2, 46.8, 47.1, 47.6, 66.3, 79.7, 119.6, 124.4, 126.8, 127.4, 127.8, 128.2, 136.4, 141.1, 143.7, 155.6, 156.3, 172.7; m/z (ES) 587 (MH<sup>+</sup>); (HRES) MH<sup>+</sup>, found 587.3129. C<sub>35</sub>H<sub>43</sub>N<sub>2</sub>O<sub>6</sub><sup>+</sup> requires 587.3116.

**4.2.13. Compound (22).** To a solution of **21** (150 mg, 0.26 mmol) in dry MeOH (9 mL), acetic acid (29 μL, 0.512 mmol) and palladium on charcoal (10% w/w, 15 mg) were added. The reaction mixture was stirred under a hydrogen atmosphere at room temperature for 1 h and filtered through Celite. The solvent was evaporated in vacuo to give crude **22** (118 mg, 100%, colorless oil), which was used in the next step without purification; R<sub>f</sub> (95:5 CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>OH) 0.13; δ<sub>H</sub> (300.10 MHz, CDCl<sub>3</sub>, mixture of rotamers) 1.05–1.60 (15H, m, CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>N, COOC(CH<sub>3</sub>)<sub>3</sub>), 2.15 (2H, t, J 6.0 Hz, CH<sub>2</sub>COO), 2.60 (0.6H, br s, CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>N), 2.90–3.20 (4H, CONHCH<sub>2</sub>CH<sub>2</sub>N, CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>N, CONHCH<sub>2</sub>CH<sub>2</sub>N and CONHCH<sub>2</sub>CH<sub>2</sub>N), 3.38 (1.4H, br s, CONHCH<sub>2</sub>CH<sub>2</sub>N), 4.19 (1H, br s, CH<sub>2</sub>CHFmoc), 4.52 (2H, m, CH<sub>2</sub>CHFmoc), 7.38 (4H, m, Ar. (Fmoc)), 7.54 (2H, d, J 7.0 Hz, Ar. (Fmoc)), 7.74 (2H, t, J 7.0 Hz, Ar. (Fmoc)); δ<sub>C</sub> (100.03 MHz, CDCl<sub>3</sub>) 22.6, 24.4, 24.7, 25.9, 26.1, 28.1, 35.1, 25.4, 38.8, 42.4, 46.5, 47.3, 47.9, 53.4, 67.0, 80.1, 119.8, 119.9, 124.0, 124.6, 126.9, 127.1, 127.7, 140.5, 141.3, 143.8, 149.0, 155.8, 157.1, 172.7, 172.9; m/z (ES) 453 (MH<sup>+</sup>); (HRES) MH<sup>+</sup>, found 453.2740. C<sub>27</sub>H<sub>37</sub>N<sub>2</sub>O<sub>4</sub><sup>+</sup> requires 453.2748.

**4.2.14. Compound (23).** To a solution of **22** (115 mg, 0.26 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (5 mL), ethyl glyoxalate (33 μL, 0.33 mmol), Et<sub>3</sub>N (54 μL, 0.38 mmol), and NaHB(OAc)<sub>3</sub> (109 mg, 0.52 mmol) were added. The reaction mixture was stirred for 18 h. The resulting mixture was washed with an aqueous saturated solution of NaHCO<sub>3</sub> and the aqueous phase extracted with CH<sub>2</sub>Cl<sub>2</sub> (3 × 10 mL). The organic phase was dried over MgSO<sub>4</sub>, filtered, and the solvent evaporated in vacuo to give the crude product, which was purified by flash chromatography (AcOEt/petroleum ether from 60:40 to 100:0) to give **23** (35 mg, 25%) as white light oil. Found: C, 69.2; H, 7.9. C<sub>31</sub>H<sub>42</sub>N<sub>2</sub>O<sub>6</sub> requires C, 69.12; H, 7.86%; R<sub>f</sub> (95:5:0.1 CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>OH/NH<sub>3</sub> 2.0 M solution in ethyl alcohol) 0.46; δ<sub>H</sub> (300.10 MHz, CDCl<sub>3</sub>, mixture of rotamers) 1.00–1.80 (18H, m, CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>N, COOC(CH<sub>3</sub>)<sub>3</sub> and COOCH<sub>2</sub>CH<sub>3</sub>), 2.18 (2H, t, J 6.0 Hz, CH<sub>2</sub>COOC(CH<sub>3</sub>)<sub>3</sub>), 2.46 (0.8H, br s, CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>N), 2.74 (1.2H, br s, CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>N), 2.90–3.45 (6H, m, COCH<sub>2</sub>NHCH<sub>2</sub>CH<sub>2</sub>N), 4.18–4.23 (3H, m, COOCH<sub>2</sub>CH<sub>3</sub>, CH<sub>2</sub>CHFmoc), 4.52 (2H, m, CH<sub>2</sub>CHFmoc), 7.31 (2H, t, J

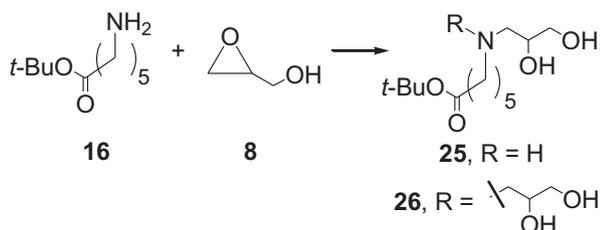
7 Hz, Ar. (Fmoc)), 7.39 (2H, t, J 7 Hz, Ar. (Fmoc)), 7.57 (2H, d, J 7 Hz, Ar. (Fmoc)), 7.75 (2H, t, J 7 Hz, Ar. (Fmoc)); δ<sub>C</sub> (100.03 MHz, CDCl<sub>3</sub>, mixture of rotamers) 14.1, 24.7, 26.1, 28.0, 35.3, 46.8, 47.3, 47.8, 50.6, 60.7, 66.5, 79.9, 119.8, 124.6, 126.9, 127.5, 141.3, 144.0, 155.9, 156.2, 172.2, 172.9; m/z (ES) 539 (MH<sup>+</sup>); (HRES) MH<sup>+</sup>, found 539.3108. C<sub>31</sub>H<sub>43</sub>N<sub>2</sub>O<sub>6</sub><sup>+</sup> requires 539.3116.

**4.2.15. Compound (24).** To a solution of **23** (100 mg, 0.186 mmol) in DMF (6 mL), thymine-1-acetic acid (55 mg, 0.30 mmol), PyBOP (154 mg, 0.30 mmol), and triethylamine (83 μL, 0.60 mmol) were added. The reaction mixture was stirred for 18 h, concentrated in vacuo, dissolved in CH<sub>2</sub>Cl<sub>2</sub> (20 mL), and washed 1 M HCl solution. The aqueous layer was extracted with CH<sub>2</sub>Cl<sub>2</sub> (3 × 20 mL). The combined organic phases were dried over MgSO<sub>4</sub>, filtered, and the solvent evaporated in vacuo to give a crude material, which was purified by flash chromatography (AcOEt/petroleum ether from 30:70 to 100:0) to give **24** (92 mg, 70%) as amorphous white solid. Found: C, 64.7; H, 6.9. C<sub>38</sub>H<sub>48</sub>N<sub>4</sub>O<sub>9</sub> requires C, 64.76; H, 6.86%; R<sub>f</sub> (6:40 AcOEt/petroleum ether) 0.11; δ<sub>H</sub> (250.13 MHz, CDCl<sub>3</sub>, mixture of rotamers) 1.00–1.60 (18H, m, CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>N, COOC(CH<sub>3</sub>)<sub>3</sub> and COOCH<sub>2</sub>CH<sub>3</sub>), 1.89 (3H, s, CH<sub>3</sub>-thymine), 2.18 (2H, m, CH<sub>2</sub>COOC(CH<sub>3</sub>)<sub>3</sub>), 2.95–3.65 (6H, m, NHCH<sub>2</sub>CH<sub>2</sub>NCH<sub>2</sub>), 4.00–4.80 (9H, m, CH<sub>2</sub>OOCCH<sub>2</sub>NHCH<sub>2</sub>, CH<sub>2</sub>CHFmoc and CH<sub>2</sub>-thymine), 6.94–7.00 (1H, complex signal, CH-thymine), 7.39 (2H, t, J 7.0 Hz, Ar. (Fmoc)), 7.42 (2H, t, J 7.0 Hz, Ar. (Fmoc)), 7.56 (2H, d, J 7.0 Hz, Ar. (Fmoc)), 7.76 (2H, t, J 7.0 Hz, Ar. (Fmoc)), 8.43 (1H, br s, NH-thymine); δ<sub>C</sub> (75.50 MHz, CDCl<sub>3</sub>, mixture of rotamers) 12.1, 13.9, 24.6, 26.0, 28.0, 35.3, 46.4, 46.7, 47.2, 47.8, 48.1, 50.7, 61.7, 66.9, 80.1, 110.6, 111.0, 119.9, 124.6, 127.0, 127.6, 141.3, 143.8, 143.9, 151.2, 156.4, 164.3, 167.7, 168.9, 173.0; m/z (ES) 705 (MH<sup>+</sup>); (HRES) MH<sup>+</sup>, found 705.3506. C<sub>38</sub>H<sub>49</sub>N<sub>4</sub>O<sub>9</sub><sup>+</sup> requires 705.3494.

**4.2.16. Compound (2).** To a solution of **24** (175 mg, 0.25 mmol) in a 1:1 mixture of 1,4-dioxane/water (6 mL) at 0 °C, LiOH monohydrate (23 mg, 0.55 mmol) was added. The reaction mixture was stirred for 30 min and saturated solution of NaHSO<sub>4</sub> added until pH ~3. The aqueous layer was extracted with CH<sub>2</sub>Cl<sub>2</sub> (3 × 10 mL) and once with AcOEt. The combined organic phases were dried over MgSO<sub>4</sub>, filtered, and the solvent evaporated in vacuo to give a crude material, which was purified by flash chromatography (CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>OH from 95:5 to 80:20) to give **2** (50 mg, 30%) as amorphous white solid. Found: C, 64.0; H, 6.6. C<sub>36</sub>H<sub>44</sub>N<sub>4</sub>O<sub>9</sub> requires C, 63.89; H, 6.55%; R<sub>f</sub> (9:1:0.1 CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>OH/NH<sub>3</sub> 2.0 M solution in ethyl alcohol) 0.22; δ<sub>H</sub> (400.13 MHz, CDCl<sub>3</sub>, mixture of rotamers) 1.00–1.50 (15H, m, CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>N and COOC(CH<sub>3</sub>)<sub>3</sub>), 1.86 (3H, s, CH<sub>3</sub>-thymine), 2.17 (2H, m, J 6.0 Hz, CH<sub>2</sub>COOC(CH<sub>3</sub>)<sub>3</sub>), 2.90–3.70 (6H, m, NHCH<sub>2</sub>CH<sub>2</sub>NCH<sub>2</sub>), 3.90–4.85 (7H, m, OCCH<sub>2</sub>NHCH<sub>2</sub>, CH<sub>2</sub>CHFmoc and CH<sub>2</sub>-thymine), 7.02 (1H, br s, CH-thymine), 7.10–7.45 (4H, m, Ar. (Fmoc)), 7.57 (2H, d, J 7.0 Hz, Ar. (Fmoc)), 7.77 (2H, t, J 7.0 Hz, Ar. (Fmoc)); 10.00 (1H, br s, NH-thymine); δ<sub>C</sub> (100.03 MHz, CDCl<sub>3</sub>, mixture of rotamers) 13.5, 22.7, 25.9, 26.0, 27.3, 28.8, 29.4, 29.7, 36.6, 36.7, 45.8, 47.8, 48.5, 48.8, 49.6, 54.7, 68.3, 81.4, 81.6, 111.8, 111.9, 121.2, 125.9, 126.5, 128.3, 129.0, 129.5, 130.3, 139.1, 142.6, 142.9, 145.0, 145.1, 152.6, 157.7, 166.2, 169.0, 172.7, 174.4; m/z (ES) 677 (MH<sup>+</sup>); (HRES) MH<sup>+</sup>, found 677.3182. C<sub>36</sub>H<sub>45</sub>N<sub>4</sub>O<sub>9</sub><sup>+</sup> requires 677.3181.

**4.2.17. Low yield synthesis of tert-butyl 6-(2,3-dihydroxypropylamino) hexanoate (25) and unwanted tert-butyl 6-(bis(2,3-dihydroxypropyl) amino)hexanoate (26).** To a solution of glycidol (**8**, 134 μL, 2.02 mmol) in DMF (3 mL), tert-butyl 6-aminohexanoate (**16**, 456 mg, 2.44 mmol) in DMF (3 mL), and DIPEA (0.55 mL, 3.17 mmol) were added (Scheme 3). The reaction mixture was refluxed for 3 days. NaHCO<sub>3</sub> (320 mg, 1.79 mmol) was added and the solvent was concentrated in vacuo to give the crude product, which was purified by flash chromatography (CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>OH/NH<sub>3</sub>

2.0 M solution in ethyl alcohol from 100:0:0.1 to 88:12:0.1) to give **25** (60 mg, 11%) and **26** (320 mg, 47%). Compound **25**: pale yellow oil. Found: C, 59.8; H, 10.5.  $C_{13}H_{27}NO_4$  requires C, 59.74; H, 10.41%;  $R_f$  (90:10:0.1  $CH_2Cl_2/CH_3OH/NH_3$  2.0 M solution in ethyl alcohol) 0.31;  $\delta_H$  (300.10 MHz,  $CDCl_3$ ) 1.31 (2H, q,  $J$  6.5 Hz,  $CH_2CH_2CH_2COO$ -Bu), 1.43 (9H, s,  $COOC(CH_3)_3$ ), 1.52 (2H, quint,  $J$  6.5 Hz,  $CH_2CH_2CH_2NH$ ), 1.57 (2H, q,  $J$  6.5 Hz,  $CH_2CH_2CH_2COO$ -Bu), 2.20 (2H, t,  $J$  6.5 Hz,  $CH_2CH_2CH_2COO$ -Bu), 2.58 (2H, m,  $CH_2NHCH_2CH(OH)CH_2(OH)$ ), 2.69 (1H, dd,  $J$  15.0, 6.0 Hz,  $NHCHHCH(OH)CH_2(OH)$ ), 2.80 (1H, dd,  $J$  15.0, 3.0 Hz,  $CHHCH(OH)CH_2(OH)$ ), 3.59 (1H, dd,  $J$  9.0, 3.0 Hz,  $CH_2CH(OH)CHH(OH)$ ), 3.70 (1H, dd,  $J$  9.0, 3.0 Hz,  $CH_2CH(OH)CHH(OH)$ ), 3.77 (1H, m,  $CH_2CH(OH)CH_2(OH)$ );  $\delta_C$  (62.89 MHz,  $CDCl_3$ ) 24.6, 26.4, 27.9, 28.7, 35.2, 49.2, 51.8, 65.1, 69.7, 80.0, 173.0;  $m/z$  (ES) 262 ( $MH^+$ ); (HRES)  $MH^+$ , found 262.2017.  $C_{13}H_{28}NO_4^+$  requires 262.2013. Compound **26**: yellow oil. Found: C, 57.3; H, 9.8.  $C_{16}H_{33}NO_6$  requires C, 57.29; H, 9.92%;  $R_f$  (90:10:0.1  $CH_2Cl_2/CH_3OH/NH_3$  2.0 M solution in ethyl alcohol) 0.44;  $\delta_H$  (400.13 MHz,  $CDCl_3$ , mixture of diastereoisomers) 1.27 (2H, q,  $J$  6.5 Hz,  $CH_2CH_2CH_2COO$ -Bu), 1.43 (11H, m,  $COOC(CH_3)_3$  and  $CH_2CH_2CH_2NH$ ), 1.57 (2H, q,  $J$  6.5 Hz,  $CH_2CH_2CH_2COO$ -Bu), 2.19 (2H, t,  $J$  6.5 Hz,  $CH_2CH_2CH_2COO$ -Bu), 2.40–2.60 (6H, m,  $CH_2NH[CH_2CH(OH)CH_2(OH)]_2$ ), 3.50 (2H, m,  $NH[CH_2CH(OH)CHH(OH)]_2$ ), 3.63 (2H, m,  $NH[CH_2CH(OH)CHH(OH)]_2$ ), 3.77 (2H, m,  $NH[CH_2CH(OH)CH_2(OH)]_2$ );  $\delta_C$  (62.89 MHz,  $CDCl_3$ , mixture of diastereoisomers) 26.1, 27.5, 28.0, 29.3, 29.4, 36.7, 56.8, 56.9, 58.3, 59.1, 66.0, 66.2, 70.5, 71.0, 81.5, 174.6;  $m/z$  (ES) 336 ( $MH^+$ ); (HRES)  $MH^+$ , found 336.2395.  $C_{16}H_{34}NO_6^+$  requires 336.2381.



Scheme 3. Attempted synthesis of compound 25.

### 4.3. General procedure for manual solid-phase oligomerization

PNA oligomers were assembled on a Rink amide PEGA resin using the above obtained Fmoc-protected PNA modified monomers as well as normal PNA monomers.

Rink amide PEGA resin (50–100 mg) was first swelled in  $CH_2Cl_2$  for 30 min. Normal PNA monomers (from Link Technologies) were provided by Advance Biosystem Italia srl. The Fmoc group was then deprotected by 20% piperidine in DMF (8 min  $\times$  2). The resin was washed with DMF and  $CH_2Cl_2$  and tested to be positive by the Kaiser test. The resin was preloaded with an Fmoc-glycine and subsequently, the coupling of the monomers (PNA or PNA modified) was conducted with either one of the following two methods (A and B). Method A: monomer (5 equiv), HBTU (4.9 equiv), and DIPEA (10 equiv); method B: modified monomer (2 equiv), HATU (2 equiv), and DIPEA (10 equiv). When the monomer was coupled to a primary amine, i.e., to a classic PNA monomer, method A was used; when the coupling was to a secondary amine, i.e., to a modified PNA monomer, method B was used. The coupling mixture was added directly to the Fmoc-deprotected resin. The coupling reaction required 30 min at room temperature for the introduction of both normal and modified monomers, in case of method B the coupling time was raised to 6 h, and the resin was then washed by DMF/ $CH_2Cl_2$ . The Fmoc deprotection and monomer coupling cycles were continued until the coupling of the last residue. After every

coupling the unreacted sites were capped by adding 5% acetic anhydride and 6% DIPEA in DMF and the reaction vessel was shaken for 1 min (twice), and subsequently washed with a solution of 5% of DIPEA in DMF. The resin was always washed thoroughly with DMF/ $CH_2Cl_2$ . The cleavage from the resin was achieved by addition of a 10% solution of *m*-cresol in TFA. The PNA was then precipitated adding 10 volumes of diethyl ether, cooled at  $-18^\circ C$  for at least 2 h and finally collected through centrifugation (5 min@5000 rpm). The resulting residue was redissolved in  $H_2O$  and purified by semipreparative reverse-phase C18 (Waters, Bondapak, 10  $\mu m$ , 125  $\text{\AA}$ ,  $7.8 \times 300$  mm) gradient elution from 100%  $H_2O$  (0.1% TFA, eluent A) to 60%  $CH_3CN$  (0.1% TFA, eluent B) in 30 min. The product was then lyophilized to get a white solid. MALDI-TOF MS analysis confirmed the expected structures for the four oligomers (**3–6**), with peaks at the following  $m/z$  values: compound **3**  $m/z$  (MALDI-TOF MS—negative mode) 2839 ( $M-H$ ) $^-$ , calcd for  $C_{112}H_{140}N_{59}O_{33}$  2839.11; compound **4**  $m/z$  (MALDI-TOF MS—negative mode) 2955 ( $M-H$ ) $^-$ , calcd for  $C_{116}H_{144}N_{59}O_{37}$  2955.12; compound **5**  $m/z$  (MALDI-TOF MS—negative mode) 2895 ( $M-H$ ) $^-$ , calcd for  $C_{116}H_{148}N_{59}O_{33}$  2895.17; compound **6**  $m/z$  (MALDI-TOF MS—negative mode) 3123 ( $M-H$ ) $^-$ , calcd for  $C_{128}H_{168}N_{59}O_{37}$  3123.31.

### 4.4. Thermal denaturation studies

DNA oligonucleotides were purchased from CEINGE Biotecnologie avanzate s.c. a.r.l.

The concentrations of PNAs were quantified by measuring the absorbance ( $A_{260}$ ) of the PNA solution at 260 nm. The values for the molar extinction coefficients ( $\epsilon_{260}$ ) of the individual bases are:  $\epsilon_{260}$  (A)=13.7 mL/( $\mu mol \times cm$ ),  $\epsilon_{260}$  (C)=6.6 mL/( $\mu mol \times cm$ ),  $\epsilon_{260}$  (G)=11.7 mL/( $\mu mol \times cm$ ),  $\epsilon_{260}$  (T)=8.6 mL/( $\mu mol \times cm$ ) and molar extinction coefficient of PNA was calculated as the sum of these values according to sequence.

The PNA oligomers and DNA were hybridized in a buffer 100 mM NaCl, 10 mM sodium phosphate, and 0.1 mM EDTA, pH 7.0. The concentrations of DNA and modified PNA oligomers were 5  $\mu M$  each for duplex formation. The samples were first heated to  $90^\circ C$  for 5 min, followed by gradually cooling to room temperature. Thermal denaturation profiles (abs vs  $T$ ) of the hybrids were measured at 260 nm with an UV/Vis Lambda Bio 20 Spectrophotometer equipped with a Peltier Temperature Programmer PTP6 interfaced to a personal computer. UV-absorption was monitored at 260 nm from an 18– $90^\circ C$  range at the rate of  $1^\circ C$  per minute. A melting curve was recorded for each duplex. The melting temperature ( $T_m$ ) was determined from the maximum of the first derivative of the melting curves.

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